Immune responses to and adjuvant properties of bacterial capsular polysaccharides

By

Hannah Elizabeth Jones

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The Edward Jenner Institute for Vaccine Research

University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Complement plays an important role in the humoral response to protein antigens, but its role in anti-capsular antibody responses is unclear. The immunogenicity of a panel of twelve CPSs from *S. pneumoniae* was investigated in mice deficient in complement receptors 1 and 2 (CD35/CD21, respectively). With the exception of serotype 4, IgM anti-polysaccharide responses were not dependent on the presence of CD35/CD21. In contrast IgG anti-polysaccharide responses, which were infrequently and variably elicited in C57BL/6 mice, were critically dependent on the presence of CD21/CD35. Although classified as T-independent type 2 (TI-2) antigens, antibody responses to pneumococcal CPSs, except serotype 4, were different from those to dinitrophenol (DNP)-Ficoll, a model TI-2 antigen. Our results indicated that complement plays a critical role in IgG anti-polysaccharide and anti-hapten responses. However IgM anti-pneumococcal CPS responses, with the only one exception, were surprisingly complement-independent. Our findings establish a differential role of complement in humoral responses to a model TI-2 antigen and clinically relevant polysaccharide antigens.

In addition to the studies of *S. pneumoniae* CPSs, the adjuvant properties of CPSs from *K. pneumoniae* were also invesitgated. Several *K. pneumoniae* CPS preparations, in particular K1 and K3, have been reported to possess potent immunomodulatory properties. However the purity of CPSs used was not investigated adequately. We modified previously established methods of CPS purification in order to obtain highly purified CPSs for our studies. Pure

CPSs with molecular weights of 1-2 x 10^6 daltons failed to augment the antibody response to chicken gamma globulin (CGG) when used as an adjuvant in mice. *In vitro* studies also demonstrated that crude but not highly purified CPSs induced the proliferation of or cytokine production from splenocytes from normal and LPS-hyporesponsive mice. The active component (s) in crude CPSs has not been identified, but it is resistant to heat, protease, nuclease and alkaline treatments. We have also ruled out the possibility that the immunomodulatory effects were due to bacterial DNA or LPS, two common contaminants in these preparations. Since not all the components in CPS extracts from *K. pneumoniae* have been characterised, further investigations may identify novel adjuvant candidate (s).

Publications and Abstracts

Publications

1) Zamze,S., Martinez-Pomares, L., **Jones, H.**, Taylor, P. R., Stillion, R. J., Gordon, S. and Wong, S. Y. (2002) Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor *J. Biol Chem,* 277, 41613-23.

2) **Jones, H.**, Taylor P. R., Carroll, M. C., Wong, S. Y. and Zamze, S. Differential dependency on complement C3 and complement receptors CD21/CD35 in murine antibody responses toward pneumococcal capsular polysaccharides and a model T-independent type II antigen. (Manuscript in preparation)

Abstracts

1) **Jones, H.**, Taylor P. R., Carroll, M. C., Wong, S. Y. and Zamze, S. The role of complement in the humoral immune response to capsular polysaccharides from *Streptococcus pneumoniae.* Abstract was selected for oral presentation by H. Jones at the 12th International Congress of Immunology, Montreal, Canada, 2004.

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Abbreviations

ANOVA	Analysis of variance
APC	Antigen presenting cell
BCR	B cell receptor
BSA	Bovine serum albumin
CD21	Complement receptor 2
CD35	Complement receptor 1
CGG	Chicken gamma globulin
CPS	Capsular polysaccharide
CRD	Carbohydrate recognition domain
DC	Dendritic cell
FCS	Foetal calf serum
FDC	Follicular dendritic cell
D-Fuc	Fucose
FucNAc	*N*-acetyl-fucosamine
D-Gal	Galactose
GalA	Galacturonic acid
GalNAc	*N*-acetyl-galactosamine
D-Glc	Glucose
GlcA	Gluronic acid
GlcNAc	*N*-acetylglucosamine
h	hour
HPLC	High pressure liquid chromatography
Ig	Immunoglobulin
i.p	intraperitoneal
L	litre
LAL	Limulus amoebocyte lysate
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
D-Man	Mannose
ManNAc	*N*-acetylmannosamine
MBL	Mannose binding lectin
mins	minutes
MR	Mannose receptor
MHC	Major histocompatibility complex
MWCO	Molecular weight cut off
MZ	Marginal Zone
MZ B	Marginal Zone B cells
MZM	Marginal Zone macrophages
MZMM	Marginal Zone metallophilic macrophages
OAc	O-acetyl
PBS	Phosphate buffered saline
PMA	Phorbol myristate acetate
L-Rha	Rhamnose
L-Rib	Ribose
SEM	Standard error of the mean
TA	Teichoic acid
TCR	T cell receptor

TD	T-dependent
TI	T-independent
TLR	Toll-like receptor

Chapter One

Introduction

Chapter One

Introduction

1.1 General Introduction

Polysaccharide capsules of encapsulated bacteria, such as *Haemophilus influenzae* and *Streptococcus pneumoniae,* can protect bacteria from innate immune mechanisms. Consequently, the capacity to generate opsonising antibody to encapsulated bacteria has been long recognised as being essential for host defence (Macleod et al., 1945). The clinical importance of antibody responses to the capsular polysaccharide (CPS) of these bacteria is highlighted in the high rate of mortality and morbidity from these pathogens during infancy. The T-independent type II nature of these antigens results in a lack of antibody production in the very young and those who are immunocompromised. Polysaccharide-only vaccines are thus ineffective in these populations (Mond et al., 1995b). The requirement for effective vaccines for infants is therefore crucial. Polysaccharide-protein conjugate vaccines have been shown to elicit primary and memory humoral responses in young children and thus have great potential as vaccines of choice against encapsulated bacteria. Whilst differences in the responses to polysaccharide and polysaccharide-protein conjugate vaccines are attributable to their ability in recruiting T cell help the mechanism of induction of anti-polysaccharide antibodies have not been well defined for either vaccine (Lindberg, 1999).

The understanding of the immune response to encapsulated bacteria has increased over the last few years and the importance of specialised B cell populations, marginal zone B cells and peritoneal B1 cells, in the generation of antibodies to polysaccharides has been established (Martin et al., 2001). The innate immune system is also well equipped to recognise microbial polysaccharides and is thus critical in mounting initial defence mechanisms through activation of complement *via* the alternative and lectin pathways, recognition by surface and soluble receptors and the presence of natural IgM antibodies. The link between innate and adaptive immunity is also of particular interest; for example the interactions between polysaccharides and complement may influence subsequent humoral responses.

Further studies to characterise the link between the innate and adaptive immune system and to define mechanisms for enhancing antibody responses against polysaccharides may provide novel strategies for the production of vaccines against multiple serotypes of encapsulated bacteria. One of the main aims of this thesis was to establish the humoral responses in normal mice to a panel of capsular polysaccharides from *S. pneumoniae,* and then compare them to the response in mice deficient in complement receptors 1 and 2. From these results the differences in immunogenicity of CPS and the role of complement receptors in the immune response to different CPSs could be determined. By studying possible mechanisms involved in antibody production to polysaccharides, it may be possible to improve vaccine formulations or understand better their mechanism of action.

As well as identifying factors that determine or influence immunogenicity of capsular polysaccharides, another aspect of this thesis was to investigate polysaccharides as possible adjuvants. Certain polysaccharides of microbial origin have been described as having potent immunomodulating properties (Tzianabos., 2000). An example is β-glucan, purified from yeast and fungi. It has been shown to exhibit a wide range of biological functions, including anti-tumour activity and non specific activation of cellular and humoral components of the immune system (Bleicher et al., 1995). Possible immunostimulatory properties have also been described for certain CPS preparations from *Klebsiella pneumoniae* (Choy et al., 1996; Ho et al., 2000), although these studies are far fewer and less extensive than those for β-glucans. The other main aim of the thesis was to characterise CPS preparations from *K. pneumoniae* and determine whether CPS itself was responsible for any immunostimulatory or adjuvant effects observed. The development of new adjuvants is a major goal in vaccine development as currently only alum is licensed for use in humans. It is therefore important that studies are carried out to discover new adjuvants. Polysaccharides tend to be relatively non toxic and stable. If adjuvant properties can be attributed to these molecules they could provide a good and potentially safe adjuvant for use in vaccines.

This thesis was thus aimed to characterise factors that affect the humoral response towards CPS and to understand further the adjuvant properties of CPS preparations from *K. pneumoniae.*

1.2 Bacterial Polysaccharides

Bacteria produce a range of complex polysaccharides which are ubiquitous components on the cell surface. Major groups include CPS, teichoic acid (TA), lipoteichoic acid (LTA), and lipopolysaccharide (LPS). CPS may be present on both Gram positive and Gram negative organisms whereas LPS is only found on Gram negative and TA and LTA on Gram positive bacteria. The location on the bacterial surface and the structural features of these polysaccharide containing molecules are shown in Figures 1.1 and 1.2, respectively.

1.2.1 Capsular polysaccharides

CPS are major cell surface antigens of both Gram negative and Gram positive bacteria (Kenne et al., 1983). The formation of the capsule around the surface of the bacteria may protect the bacteria from harmful effects of desiccation and can promote the adherence of bacteria to both surfaces and each other, and they thereby facilitate the formation of biofilms and the colonisation of ecological niches (Costerton et al., 1987; Roberts, 1996). CPS are high molecular weight polysaccharides, typically 10^6 Daltons and greater. With exception of homopolymers they are composed of linear or branched repeating units consisting of 2 – 8 monosaccharides. They are structurally very diverse and are nearly always polyanionic. CPS can also be lipidated, a modification responsible for anchoring these highly charged molecules into the bacterial outer membrane (Arakere et al., 1994).

K. pneumoniae and *S. pneumoniae* are examples of clinically relevant encapsulated bacteria. Both bacterial species are opportunistic pathogens,

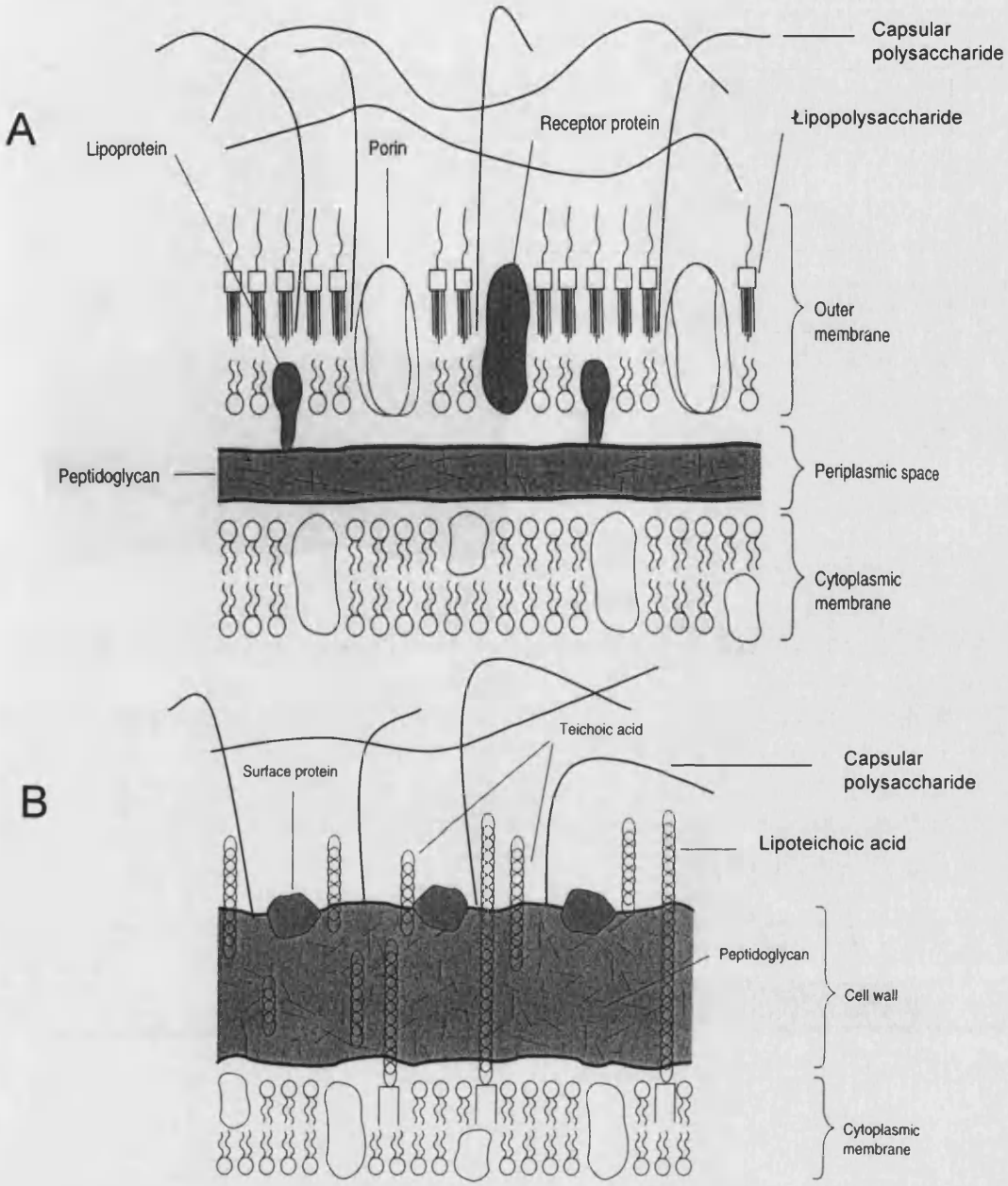
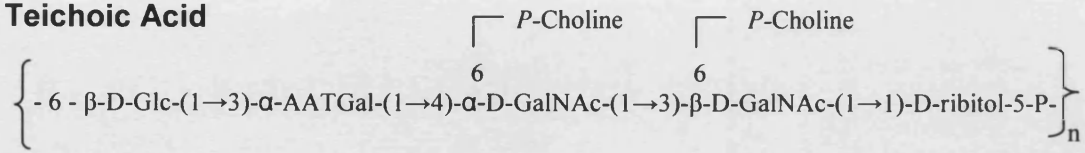


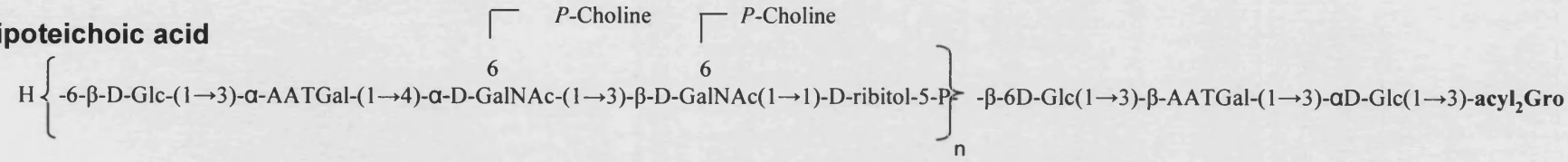
Figure 1.1 Structure of the cell surface of a Gram positive and a Gram negative bacterium.

This figure, taken from Salyers et al., 1994, illustrates the major features of a Gram negative (A) and Gram positive (B) cell surface. In particular the presence of lipopolysaccharide, lipoteichoic acid, teichoic acid and peptidoglycan. Capsular polysaccharide is also indicated on the diagram, this can be found attached to the cell surface or as 'free' polysaccharide.

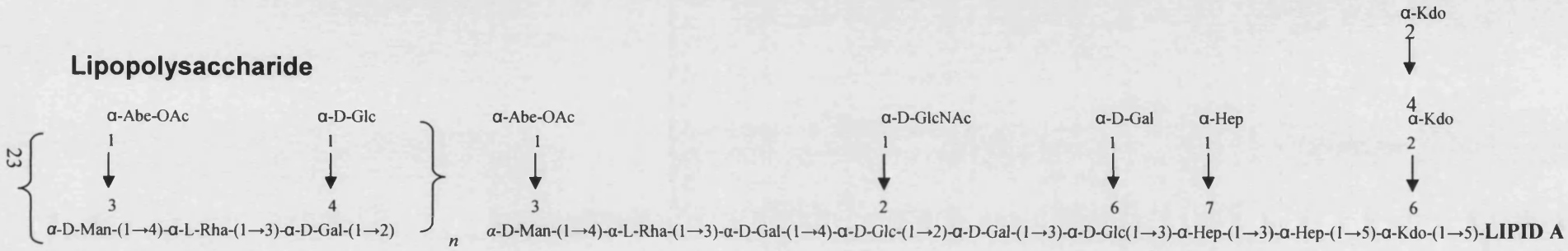
Teichoic Acid

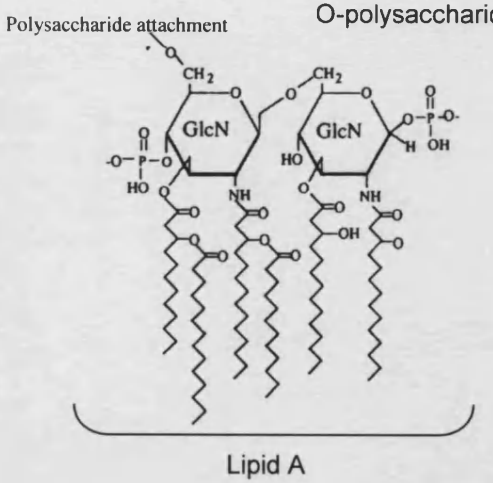


Lipoteichoic acid



Lipopolysaccharide





Capsular Polysaccharide

$$\left\{ \rightarrow 3)\text{-}\beta\text{-D-Gal-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc-}(1\rightarrow 3)\text{-}[4,6\text{-(S)-pyruvate})\text{-}\beta\text{-D-Glc-}(1\rightarrow 2)\text{-}\alpha\text{-D-Man-}(1\rightarrow 4)]\text{-}\alpha\text{-D-GlcA-}(1\rightarrow \right\}_{n}$$

Bacterial Component	Specific features
Teichoic Acid	The cell walls of most Gram positive bacteria have teichoic acids. Teichoic acids are cell associated polysaccharides containing glycerol phosphate or ribitol phosphate residues. The teichoic acids are covalently linked to peptidoglycan, making them an integral part of the Gram positive cell wall.
Lipoteichoic acid	Lipoteichoic acid has the same structural features as teichoic acid except that it is attached to the bacterial cytoplasmic membrane through di-acyl glycerol lipid anchor.
Lipopolysaccharide	LPS is found on the surface of Gram negative bacteria. LPS is a complex molecule composed of distinct regions. The innermost portion, lipid A, anchors the LPS to the outer membrane. The polysaccharide portion of the LPS consists of a core-polysaccharide region and the O-polysaccharide. The structure of the O-polysaccharide is highly variable within one species, giving rise to the different O-antigenic serotypes.
Capsular polysaccharide	Both Gram negative and Gram positive bacteria can have a capsule. The capsule is made up of repeating units consisting 2 to 8 monosaccharides. The capsule can be covalently linked to the outer membrane by a lipid anchor in the case of Gram negative bacteria, covalently linked to the peptidoglycan in the case of Gram positive, or as free polysaccharide which is not tightly bound to the cell surface.

Figure 1.2 Structural features of bacterial surface components teichoic acid, lipoteichoic acid, lipopolysaccharide and capsular polysaccharide.

The teichoic and lipoteichoic acid repeating unit structures illustrated in this figure are those from *Streptoccocus pneumoniae* (Fischer et al., 1993). The lipopolysaccharide structure is from *Salmonella enterica* (Rietschel et al., 1994) and the capsular polysaccharide structure is from *Klebsiella pneumoniae* serotype K31 (Kenne et al., 1983).

Abbreviations : AATGal, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactopyranose; $acyl_2Gro$, di-acyl glycerol; Abe, abequose; Hep, L-glycero-D-mannoheptose; Kdo, 2-keto-3-deoxyoctulosonic. Other abbreviations are listed in the abbrevations section at the beginning of the thesis.

and the correlate of protection is the production of antibodies to the CPSs (Macleod et al., 1945, Cryz et al., 1984). Vaccines against encapsulated bacteria consist of the CPS from the pathogen, which is discussed in Section 1.5. CPSs of *K. pneumoniae* are termed the K antigen, and there are at least 90 K antigens reported. Some of these structures are shown in Figure 1.3. They commonly contain glucose (D-Glc), mannose (D-Man), galactose (D-Gal) and rhamnose (L-Rha). Fucose (L-Fuc), a common constituent of mammalian glycoproteins, is found in only a few serotypes (Orskov, 1984). Each repeating unit contains a hexuronic acid residue, which is normally glucuronic acid (D-GlcA), and is frequently substituted with pyruvate acetal and O-acetyl groups. In the case of *S. pneumoniae,* 90 serotypes have been described (Henrichsen, 1995). Some of these are shown in Figure 1.4 to illustrate diversity of CPS structures. *S. pneumoniae* CPS tend to contain D-GlcA or galacturonic acid (D-GalA) acid residues and may be substituted with O-acetyl, pyruvate acetal, and glycerol phosphate groups (Jennings, 1996). Some of the pneumococcal CPSs resemble teichoic acids, as they contain ribitol phosphate-linked repeat units. They often contain amino sugars, such as amino hexoses and also *N*-acetyl-D-mannuronic acid. D-Gal, D-Glc, and L-Rha are frequently found, but D-Man is not detected in any of the pneumococcal CPS structures.

The capsule is generally considered to be an important virulence factor in *K. pneumoniae.* The capsule is critical for the organism's ability to resist complement-mediated killing (Kabha et al., 1995), and is involved in the resistance to phagocytosis by polymorphonuclear cells by acting as a

Type	Structure
K1*	→4)-[2,3-(S)-pyruvate]-β-D-GlcA-(1→4)-α-L-Fuc-(1→3)-β-D-Glc-(1→
K3[a]*	→2)-[(4,6-(S)-pyruvate)-α-D-Man-(1→4)]-α-D-GalA-(1→3)-α-D-Man-(1→2)-α-D-Man-(1→3)-β-D-Gal-(1→
K11*	→3)-β-D-Glc-(1→3)-[(4,6-(S)-pyruvate)-α-D-Gal-(1→4)]-β-D-GlcA-(1→3)-α-D-Gal-(1→
K17*	→3)-β-L-Rha-(1→4)-β-D-Glc-(1→2)-[α-L-Rha-(1→3)]-α-L-Rha-(1→4)-α-D-GlcA-(1→
K22[b]	→4)-β-D-Glc-(1→3)-[(4-O-lactate)-β-D-GlcA-(1→6)-α-D-Glc-(1→4)]-β-D-Gal-(1→ 6 ↑ OAc
K26*	→3)-β-D-Gal-(1→2)-[(4,6-(S)-pyruvate)-β-D-Gal-(1→4)-β-D-Glc-(1→6)-α-D-Glc-(1→4)]-α-D-GlcA-(1→3)-α-D-Man-(1→2)-α-D-Man-(1→
K27*	→3)-β-D-Glc-(1→3)-?Gal-(1→3)-[β-D-Glc-(1→4)]-?Gal-(1→6)-β-D-Glc-(1→ 6 ↑ 1 β-D-GlcA
K31*	→3)-β-D-Gal-(1→3)-β-D-Glc-(1→3)-[4,6-(S)-pyruvate)-β-D-Glc-(1→2)-α-D-Man-(1→4)]-α-D-GlcA-(1→
K36	→2)-α-L-Rha-(1→3)-β-D-Gal-(1→3)-[(4,6-(S)-pyruvate)-β-D-Glc-(1→4)-β-D-GlcA-(1→2)]-α-L-Rha-(1→3)-α-L-Rha-(1→
K40[c]	→3)-α-L-Rha-(1→2)-α-L-Rha-(1→4)-α-D-GlcA-(1→2)-α-D-Man-(1→2)-α-D-Man-(1→3)-α-D-Gal-(1→
K46	→3)-α-D-Gal-(1→3)-β-D-Gal-(1→3)-[β-D-Glc-(1→3)-(4,6-(S)-pyruvate)-?Man-(1→4)]-α-D-GlcA-(1→3)-α-D-Man-(1→
K52[d]*	→3)-[α-D-Gal-(1→2)]-α-D-Gal-(1→4)-α-L-Rha-(1→3)-β-D-Gal-(1→2)-α-L-Rha-(1→4)-β-D-GlcA-(1→
K55*	→3)-β-D-Glc-(1→4)-[α-D-GlcA-(1→3)-α-D-Gal-(1→3)]-α-L-Rha-(1→ 2 ↑ OAc

Figure 1.3 Structures of *K. pneumoniae* capsular polysaccharides
The structures of some *Klebsiella* CPSs are shown to illustrate the diversity of these polysaccharides. K1 and K3 are of particular interest as they have been shown to exhibit possible adjuvant properties (Choy et al., 1996). CPSs indicated by * have been used for experiments carried out in Chapters 3, 4, and 5 of this thesis. Structures are taken from Kenne et al., 1983 except for the following: (a) Dutton et al., 1986, (b) Parolis et al., 1988, (c) Cestutti et al., 1993 and (d) Stenutz et al., 1997.

Type	Structure
1	→3)-α-AATGal-(1→4)-α-D-GalA-(1→3-α-D-GalA-(1→
3	→4)-β-D-Glc-(1→3)-β-D-GlcA-(1→
4	→3)-β-D-ManNAc-(1→3)-α-L-FucNAc-(1→3)-α-D-GalNAc-(1→4)-[2,3-(S)-pyruvate]-α-D-Gal-(1→
6B	→2)-α-D-Gal-(1→3)-α-D-Glc-(1→3)-α-L-Rha-(1→4)-D-Ribitol-(5-*P*→
9N	→4)-α-D-GlcA-(1→3)-α-D-Glc-(1→3)-β-D-ManNAc-(1→4)-β-D-Glc-(1→4)-α-D-GlcNAc-(1→
9V	→4)-α-D-GlcA-(1→3)-α-D-Gal-(1→3)-β-D-ManNAc-(1→4)-β-D-Glc-(1→4)-α-D-GlcNAc-(1→ ↑ OAc ↑ OAc
14	→4)-β-D-Glc-(1→6)-[β-D-Gal(1→4)]-β-D-GlcNAc-(1→3)-β-D-Gal-(1→
18C	→4)-β-D-Glc-(1→4)-[α-L-Glc-(1→2)]-β-D-Gal-(1→4)-α-D-Glc-(1→3)-α-L-Rha-(1→ 3 ↑ *P*-1-glycerol
19A	→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→3)-α-L-Rha-(1-*P*→
19F	→4)-β-D-ManNAc-(1→4)-α-D-Glc-(1→2)-α-L-Rha-(1-*P*→
23F	→4)-β-D-Glc-(1→4)-[α-L-Rha(1→2)]-β-D-Gal-(1→4)-β-L-Rha-(1→ 3 ↑ *P*-2-glycerol
CW-PS	→6)-β-D-Glc-(1→3)-α-D-AATGal-(1→4)-α-D-GalNAc-(1→3)-β-D-GalNAc-(1→1)-D-Ribitol-(5-*P*→ 6 6 ↑ ↑ *P*-choline *P*-choline

Figure 1.4 Structures of *S. pneumoniae* capsular polysaccharides
The capsular polysaccharide structures illustrated are those from serotypes associated with clinical isolates found in developed countries and are components of the 23-valent polysaccharide vaccine. These polysaccharides were used in studies carried out in Chapter 6 of this thesis. Structures are taken from Jennings et al., 1996. All sugars are in the pyranose conformation.

physical barrier. The CPS from *Klebsiella* envelopes the bacterial cell surface and prevents cell surface components such as LPS and porins from activating complement (Alvarez et al., 2000). The capsule of *S. pneumoniae* is a prerequisite for virulence as non-encapsulated mutants are quickly cleared from the circulation (Watson and Musher, 1990; Watson et al., 1995). The main role of the capsule in virulence is probably to protect the pneumococcus from phagocytosis. *Klebsiella* and pneumococcal CPSs are therefore a major target for both the innate and adaptive immune system.

CPSs are possible targets for lectin interaction resulting in lectinophagocytosis (antibody-independent phagocytosis of micro organisms mediated by lectin interaction). It has been suggested that CPS can promote binding of *K. pneumoniae* to guinea pig alveolar macrophages in serum-free medium and that binding is followed by ingestion and killing of the bacteria (Athamna et al., 1991). It has also been suggested that macrophage mannose receptor is involved (Kabha et al., 1995). The binding of purified CPSs to the macrophage mannose receptor *in vitro* has recently been studied in our laboratory. Ten out of the twelve pneumococcal CPSs tested bound to the mannose receptor, despite structural variation. In contrast, *Klebsiella* CPSs failed to bind (Zamze et al., 2002). Interestingly *Klebsiella* LPS did bind to the mannose receptor which suggests that lectinophagocytosis is mediated through the binding of LPS, rather than CPS, to the mannose receptor. There are no obvious structural differences to explain the preferential binding of the mannose receptor to pneumococcal CPSs. Recogniton of structurally variable *Klebsiella* LPS and pneumococcal

CPSs by the mannose receptor shows that criteria other than primary polysaccharide structure (e.g CPS conformation) must be taken into consideration for lectin receptor specificity.

There have also been several reports describing the possible adjuvant activity of *Klebsiella* CPSs independent of LPS. Yokochi and colleagues showed that *Klebsiella* CPSs augmented the antibody response to various antigens in mice (Yokochi et al., 1980b). Further studies in mice have shown that *Klebsiella* CPSs induced the release of TNF-α (Choy et al., 1996, Ho et al., 2000).

1.2.2 Lipopolysaccharide

LPS is found on the outer membrane of all Gram negative bacteria as shown in Figure 1.1 (Rietschel et al., 1994). Most of our understanding of LPS has come from studies of Enterobacteriaceae. LPS in these bacteria is composed of a hydrophobic domain known as lipid A, a core oligosaccharide region and a distal polysaccharide known as the O-specific polysaccharide. If the O-specific polysaccharide is absent, the LPS is known as a rough LPS. If O-specific polysaccharide is present the LPS is referred to as smooth LPS. The O-specific polysaccharide diversity is impressive; more than 60 monosaccharides and 30 non carbohydrate components have been recognised (Raetz and Whitfield, 2002). Different Gram negative bacterial species can therefore have different O-specific polysaccharide structures. Lipid A, also known as endotoxin, is a diglucosamine-based phospholipid. Lipid A is the most conserved part of the LPS molecule and is responsible for

some of the biological activities of LPS. The inner core oligosaccharide regions connect the lipid A and O-specific polysaccharide.

Some Gram negative bacteria, such as *Haemophilus influenzae and Neisseria meningitides,* express lipooligosaccharide (LOS), which is analogous to the LPS found on other Gram negative families (Kahler et al., 1998; Swords et al., 2003). LOSs share similar lipid A and functional activities to LPSs, except LOSs lack O-specific polysaccharide units. LOS structures are limited to 10 saccharide units. LOS is predominantly expressed on mucosal bacteria whereas LPS is expressed mostly on enteric bacteria. The O-specific polysaccharide of LPS results in the bacterial cell having a hydrophilic surface. This results in the resistance of enteric bacteria to solubilisation by enzymes and bile in the gastrointestinal tract, whereas mucosal bacteria which generally lack O-specific polysaccharide tend to have hydrophobic surfaces which are susceptible to bile (Preston et al., 1996).

K. pneumoniae is a Gram negative bacterium which produces LPS, rather than LOS. There are 9 different O-specific polysaccharides identified, with O1 being the most common among clinical isolates of *K. pneumoniae*. The O polysaccharides are composed of repeating units that contain monosaccharides, such as D-Man, D-Gal and L-Rha and non-carbohydrate substituents such as O acetyl groups. Some are termed homopolymers such as O5 which has a mannose polymer, whereas those that contain more than one sugar, such as O4, are termed heteropolymers. Figure 1.5 shows

O-serotype		Structures
01	Galactan I and Galactan II:	→3)-β-Gal*f*-(1→3)-α-D-Gal-(1→ and →3)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→, respectively.
02a	Galactan I	
03	Polymannose:	→3)-α-D-Man-(1→3)-α-D-Man-(1→2)-α-D-Man-(1→2)-α-D-Man-(1→2)-α-D-Man-(1→
04		→2)-β-Rib*f*-(1→4)-D-Gal*p*-(1→

Figure 1.5 Structures of the O-antigenic sidechains of *K. pneumoniae* LPS
Structures are taken from Orskov et al., 1984. The majority of LPSs expressed by isolates of *K. pneumoniae* are of the 01, 02, and 03 serotypes. The *K. pneumoniae* strains used in studies described in Chapter 3, 4, and 5 have one of these O-antigen serotypes. Of particular interest is 03, which has been shown to have greater immunomodulatory properties than other LPS types (Ohta et al., 1985). *f* = furanose *p* = pyranose

examples of four O-specific polysaccharide structures from *K. pneumoniae* LPS (Orskov et al., 1984).

Particular O-specific polysaccharide side chains of *K. pneumoniae* LPS have been shown to possess greater immunomodulatory properties than other LPS types (Ohta et al., 1985). *K. pneumoniae* O3 and O5 polysaccharides are mannose homopolymers. They have been shown to have strong adjuvant properties compared to other O-polysaccharide structures. The mechanism of action is perhaps through enhanced complement activation. Mannose homopolymers are 100 times more potent at activating complement compared to other LPS types (Kido et al., 1985).

1.2.3 Lipoteichoic and teichoic acid

The pneumococcal cell surface is composed of membrane bound LTA encased in a cell wall consisting of a multilayered network of peptidoglycan with associated TA chains (Fischer. 1997). LTA and TA are found on the surface of Gram positive bacteria (Figure 1.1). The term teichoic acid includes all cell surface associated polymers containing glycerol phosphate or ribitol phosphate residues. These polyalcohols are connected by phosphate esters and usually have other sugars and D-alanine attached. The TA of pneumococcus is either referred to as C polysaccharides or cell wall polysaccharide (CW-PS). It is evident that the repeating units of CW-PS are identical to that reported for LTA (Jennings et al., 1980, Behr et al., 1992). The LTA is hydrophobically anchored to the cytoplasmic membrane by its ester-linked fatty acids. LTA and TA are considered pneumococcal common antigens; every Gram positive bacterial species possesses its own particular

LTA and TA. In contrast to the 90 capsular serotypes described for *S. pneumoniae* which are strain specific.

1.3 Innate immunity

The innate immune system is an ancient defence mechanism which uses a limited number of germ-line encoded receptors for the recognition of microbial pathogens. This distinguishes it from the adaptive immune system which is based on receptors that are generated by somatic gene rearrangement mechanisms. The latter mechanism generates a diverse repertoire of antigen receptors with random specificities which are clonally distributed on the surface of B and T cells. Consequently, the specificity of antigen receptors for T and B cells is not predetermined.

Pathogens are characterised by specific structures called pathogen-associated molecular patterns (PAMPs) and are recognised by pattern recognition receptors (PRRs) of the innate immune system (Medzhitov and Janeway, 1997). The PRRs are found on many cells of the innate immune system including dendritic cells (DCs) and macrophages. There are several different families of PRRs such as scavenger receptors, Toll-like receptors (TLRs), C-type lectins and complement receptors which will be discussed in more detail in the following sections. There are also several soluble factors, which bind PAMPs, these include complement, mannan-binding lectin (MBL), lipopolysaccharide binding protein (LBP) and C-reactive protein.

1.3.1 Macrophages and Dendritic cells

Professional antigen presenting cells (APCs), DCs and macrophages, are specialised phagocytes that are important in the clearance of host cells and microbial components. They are found throughout the body and participate in the initial capture and processing of potential antigens (innate immunity) and then activation of specific T and B cell effector mechanisms (adaptive immunity). In addition to their efficient endocytic and phagocytic activities, APCs are potent secretory cells that induce and regulate local and systemic inflammatory and immune responses (MacMicking et al., 1997, Banchereau et al., 2000).

Macrophages recognise pathogens by surface receptors such as mannose receptor, complement receptors, and scavenger receptors. Ligation of many of the surface receptors that recognise pathogens leads to phagocytosis of the pathogen (Underhill and Ozinsky, 2002). Internalisation of the pathogen initiates an assortment of enzymatic and oxidative mechanisms that result in the killing and disposal of engulfed particles. Microbe internalisation by macrophages is normally accompanied by the production of proinflammatory signals. Recognition of microbial components by receptors such as TLRs generally results in increased costimulatory molecule expression and the production of cytokines by macrophages.

Like macrophages, DCs express cell surface receptors that recognise microbes and microbial components. Microbial stimulation acting through receptors such as TLRs on immature DCs results in their maturation. Maturation leads to reduced endocytic and phagocytic capability, enhanced

expression of costimulatory molecules, increased antigen processing and presentation, and release of cytokines and chemokines (Rescigno, 2002). Mature DCs are therefore potent activators of T cell priming and differentiation. DCs can therefore be described as cells that link innate and adaptive immunity. DCs can direct the adaptive response by cytokine release. Cytokines are key mediators of immune responses and their release can tailor the induced T cell response. IL-12, IL-18, IFNγ and IL-23 can promote Th1 responses whereas IL-4 induces a Th2 response.

1.3.2 Innate Immune Recognition of Pathogens

1.3.2a Soluble factors

A: Complement

Complement is a complex and highly regulated biological effector system. Its functions include the recognition and elimination of pathogens and altered host cells. In this context, complement is a major constituent of the innate immune system, with the ability to discriminate self from non-self and to facilitate the removal of pathogens and any other non-self antigens, through multiple proinflammatory, opsonic, phagocytic, cytotoxic and cytolytic actions (Barrington et al., 2001).

The complement system consists of more than 30 soluble and membrane proteins. Activation is required for biological activity of the system. This initiates a highly regulated cascade of interactions between complement components, with each other, with the activator, and with cell membranes.

There are three different ways to activate complement: antibody-antigen complexes and certain negatively charged structures initiate the classical pathway (Arlaud et al., 2001); the alternative pathway is initiated by interference with regulatory components by structures on microbial surfaces (Xu at al., 2001); and the mannan-binding lectin (MBL) pathway is mediated by binding of MBL (Gadjeva et al., 2004) or ficolins (Matsushita et al., 2001) to carbohydrate. Once the complement system is activated, a chain of reactions that involve proteolysis and assembly occurs, which results in the cleavage of C3. The cascade that leads to the cleavage of C3 is called the activation pathway. It is followed by the lytic pathway, during which the membrane attack complex (MAC) is formed.

Classical complement pathway

The classical pathway can be initiated by the binding of C1 directly to the pathogen surface or to antigen-antibody complexes. HIV-1 has been shown to be recognised by C1 in the absence of specific antibody (Ebenbichler et al., 1991). C1 binding to all activators of the classical pathway is mediated by its recognition subunit C1q and is believed to generate a conformational change that triggers activation of its catalytic subunit C1s-C1r-C1r-C1s comprising the two proteases C1s and C1r (Arlaud et al., 2001). After the autolytic activation of pro-enzyme C1r, C1r activates pro-enzyme C1s, and C1s cleaves complement components C4 and C2 to produce cleavage products C2b and C4b. C2b and C4b together form the C3 convertase of the classical pathway (Kerr, 1980). The C3 convertase is therefore bound to the surface of the pathogen and cleaves C3 molecules into C3a and C3b. C3b

(acts as an opsonin) binds to the surface of the pathogen and facilitates its uptake and destruction by phagocytic cells that express CR1 (complement receptor 1) which has specificity for C3b. Many of the deposited C3b fragments are subsequently degraded to iC3b and C3dg. iC3b is a major opsonin that binds to CR3 and CR4, whereas C3dg and C3d (proteolytic fragment of C3) can only engage CR2 expressed by B cells and follicular dendritic cells (FDCs). C3a is an anaphylatoxin which has potent biological activities at very low concentrations. Activation of cells through the C3a receptor (Ember and Hugli, 1997) results in a variety of proinflammatory events including increased permeability of small blood vessels and regulation of vasodilation (Kohl, 2001).

C3b can also bind to the C3 convertase to form a C5 convertase (Rawal and Pangburn, 2003). The C5 convertase cleaves C5 into C5a and C5b. C5a is a chemotatic anaphylatoxin (DiScipio et al., 1988) and C5b initiates the formation of the membrane attack complex (MAC) which helps to destroy microrganisms. C5b forms a molecular complex with C6 to form C5b-6, the addition of this to C7 creates C5b-7. C5b-7 is capable of associating and intergrating into the lipid bilayer of the bacterium (DiScipio et al., 1988, DiScipio, 1992). After C8 adds to C5b-7, transmembrane pores are formed, and these grow and circularize with the accretion of C9 to produce the final assembly that directly lyses bacteria (Scibek et al., 2002).

Alternative complement pathway

In the alternative pathway, assembly of the C3 convertase is initiated by the covalent attachment of C3b to the surface of an activator (Xu et al., 2001). Activators include a wide range of pathogens. CPSs of *S. pneumoniae* have been shown to bind covalently to C3b (Griffioen et al., 1991). The attachment of C3b is achieved through a reaction between the carbonyl of the thioester in C3b and, typically, hydroxyl groups on the activator surface (Law et al., 1979). Following this, factor B, a serine protease, binds to the activator-linked C3b to form activator-linked C3bB. The factor B present in the complex is then subject to proteolysis by factor D, another serine protease, which results in the cleavage of factor B into Ba and Bb. Ba is released and Bb remains bound to C3b forming C3bBb, which is the C3 convertase of the alternative pathway (Xu et al., 2001). The C3bBb complex catalyzes the cleavage of C3 and C5 resulting in the formation of C3a, C3b, C5a, and C5b. The following stages are the same as those described for the classical pathway where C3b acts as an opsonin and C5b initiates the formation of the MAC.

Mannose-binding lectin pathway

MBL (Figure 1.6) is associated with serine proteases known as MBL-associated serine proteases (MASPS) (Gadjeva et al., 2004). These are structurally similar to the C1s and C1r of the classical complement pathway. MBL is a member of the collectin family of proteins. In addition to MBL, the two mucosal-associated proteins, surfactant protein A and D (SP-A and SP-D) also belong to this family of proteins. Collectins share common structural

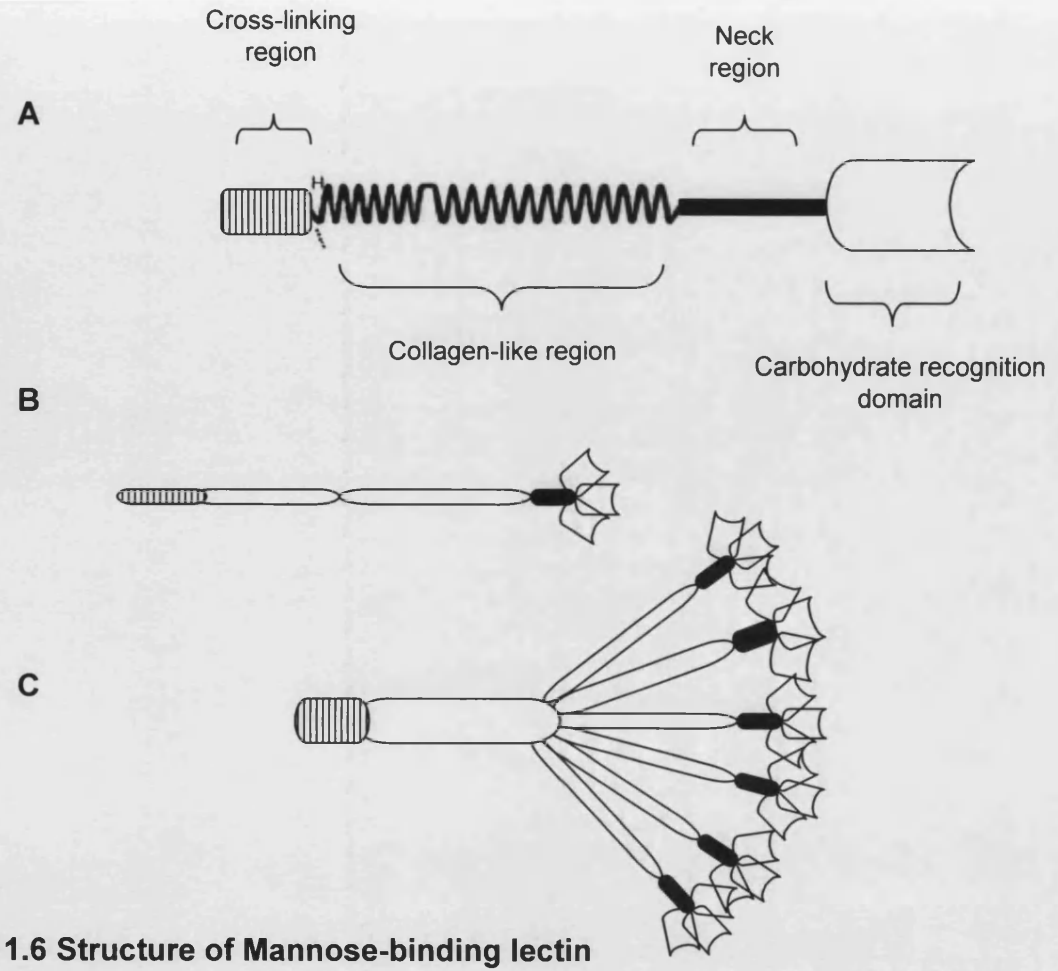


Figure 1.6 Structure of Mannose-binding lectin

This diagram was adapted from Presanis et al., 2003. The polypeptide chain of MBL is shown in (A). The neck region forms an α-helical coiled-coil structure which may promote trimerisation of three polypeptide chains as shown in (B). These trimers can assemble to form larger structures, the most common in humans is a six-subunit form which contains 18 polypeptide chains as shown in (C).

features: an amino-terminal cysteine rich region followed by a collagen-like region, an α-helical coiled region and a carboxy-terminal carbohydrate recognition domain (CRD).

MBL binds to an array of carbohydrate structures on the surface of microorganisms. For Gram negative organisms a major ligand for MBL is LPS. Lipoteichoic acids from Gram positive bacteria have also been shown to be ligands for MBL (Polotsky et al., 1996). Noncapsulated strains of *H. influenzae* and *N. meningitidis* have been shown to bind MBL better than their capsulated counterparts, suggesting the capsule can protect the bacteria from MBL (van Emmerik et al., 1994). The discrimination between self and non-self structures recognised by MBL resides in the specificity of the CRD and spatial arrangement of the CRDs. MBL preferentially recognises glycans with D-Man, D-Glu, L-Fuc, N-acetyl-mannosamine (ManNAc) and N-acetyl-glucosamine (GlcNAc) at a terminal non-reducing position (Turner, 1996). Thus MBL is very efficient at recognising microbial surfaces with high content of repetitive D-Man and/or GlcNAc such as those presented by *S. typhimurium* and *N. gonorrhoeae* (Devyatyarova-Johnson et al., 2000). Most carbohydrate structures in mammalian glycoproteins are terminated by sugars not recognised by MBL (e.g. galactose and sialic acid).

MBL bound to microbial surfaces is able to activate the complement system in an antibody independent manner. MBL-associated MASPs known as MASP-1, MASP-2 and MASP-3 have been shown to associate with MBL but evidence suggests that MASP-2 is more important in complement activation

(Matsushita and Fujita, 1992; Thiel et al., 1997; Dahl et al., 2001). Complexes of MBL and MASP-2 become activated when bound to microbial surfaces displaying specific carbohydrate ligands. MASP-2 then exhibits enzymatic activity that results in the sequential cleavage of complement components C4 and C2. C4b fragments can bind covalently to microbial surfaces which then interact with C2 components generated by MASP-2 (Turner, 1996). The C4b2a complex, is created by the cleavage of C2 and C4, has C3 convertase activity that is identical to the C3 convertase of the classical and alternative pathways. The C3 convertase can therefore generate opsonic C3b fragments.

B: C-reactive protein

C-reactive protein (CRP) is a pattern recognition molecule made up of five identical subunits; in the presence of Ca^{2+}, each subunit binds with high affinity to phosphocholine (PC) (Szalai, 2002). PC is a major constituent of the cell wall C-polysaccharide of *S. pneumoniae* and has been identified on other Gram positive bacteria such as *Clostridium* spp., *Lactococcus* spp., and *Bacillus* spp (Gillespie et al., 1996). It has also been found on the Gram negative bacteria *H. influenzae, N. meningitidis* and *N. gonorrhoeae* (Kolberg et al., 1997). The levels of CRP in the blood rise dramatically during inflammatory episodes. Ligand-bound CRP can bind to C1q and thus activate the classical complement pathway. This leads to the deposition of C3b onto the ligand-bound CRP complex, and subsequent recognition of the complex by complement receptors on phagocytes. CRP can also bind

directly to FcγRs (receptors for the Fc of IgG), resulting in the enhancement of the opsonization and phagocytosis of microbes (Stein et al., 2000).

1.3.2b Cell associated receptors

A: Scavenger receptors

Scavenger receptors (SR) is a term used to cover groups of structurally related molecules expressed on macrophages, DCs and endothelia with broad specificity for polyanionic ligands (Platt et al., 1998, Peiser et al., 2002). The following SRs have been implicated in bacterial uptake by macrophages, DCs and endothelial cells. The class A macrophage scavenger receptors are originally defined as receptors which bind to modified low-density lipoproteins (LDLs) such as oxidised LDL and acetylated LDL (Kraal et al., 2000). Three class A SRs that have been identified are SR-AI, SR-AII and MARCO (macrophage receptor with a collagenous domain). SR-A1 and SR-AII are alternative splice products of the same gene (Emi et al., 1993). The binding site for selected polyanionic ligands has been attributed to the collagenous domain. These receptors are expressed on most tissue macrophages and *in vitro* cultured DCs and can bind LTA (Dunne et al., 1994) and the lipid A part of LPS (Hampton et al., 1991). Mice deficient in SR-AI and SR-AII have been shown to be more susceptible to *L. monocytogenes* (Suzuki et al., 1997) and *S. aureus* infection (Thomas et al., 2000). MARCO is a distinct class A SR that shares structural similarity to SR-AI/SR-AII. In mice it has been shown to bind both Gram positive and Gram negative bacteria and also bind bacterial CpG DNA (Zhu et al., 2001). It is constitutively expressed on the surface of splenic marginal

zone macrophages and peritoneal macrophages (Elomaa et al., 1995) and upregulated on other macrophages by LPS (van der Laan et al., 1999).

B: C-type lectins

Cells of the immune system are equipped with many lectin and lectin-like receptors. Many of these lectins are members of the Ca^{2+}-dependent C-type lectin family and recognise ligands through the structurally related Ca^{2+}-dependent carbohydrate recognition domains (CRDs) (Drickamer. 1988, Geijtenbeek et al., 2004). Examples of these types of receptors include the mannose receptor and DC-SIGN. The mannose receptor is a pattern recognition receptor that plays an important role in the first line of host defence, by recognising carbohydrates that are present on the surface and cell walls of infectious agents. It is a 175kDa type 1 membrane molecule that has five domains: the amino-terminal cysteine rich region; a domain containing a fibronectin type II repeat; a series of 8 tandem CRDs; a transmembrane domain; and a cytoplasmic carboxy terminal domain (Taylor. 2001). It has been shown to mediate the endocytic uptake of soluble glycoconjugates and is reported to mediate lectinophagocytosis of a number of bacteria including *K. pneumoniae* (Athamna et al., 1991). The protein is expressed on macrophages and hepatic endothelial cells (Linehan et al., 1999) and DCs (*in vitro*) (Sallusto et al., 1995). Its CRDs have specificity for monosaccharides L-Fuc = D-Man > GlcNAc << D-Gal (Mullin et al., 1994). The CRDs of the mannose receptor have been shown to bind CPS from *S. pneumoniae* and LPS, but not CPS from *K. pneumoniae* (Zamze et al., 2002). It also has a role in clearance of host serum glycoproteins, lysosomal

enzymes and tissue plasminogen activator, and pituitary hormones such as lutropin and thyrotropin (Simpson et al., 1999). The mannose receptor can therefore function as both a pattern recognition receptor and a regulator of homeostasis (Allavena et al., 2004).

The mannose receptor can be found in cell bound and soluble forms. The soluble form of the receptor has been identified in both mouse and human serum. The soluble form lacks the carboxyl end of the molecule and is produced by the proteolytic activity of metalloproteinases (Martinez-Pomares and Gordon, 1999a). Recent proposals have suggested that a soluble form of the receptor could be involved in an antigen transport pathway (Martinez-Pomares and Gordon, 1999b). The discovery that there are ligands in specialised cell populations of secondary lymphoid organs, such as metallophilic macrophages from the splenic marginal zone (Martinez-Pomares et al., 1996), that are recognised by the cysteine rich domain of the mannose receptor, suggests that perhaps the soluble mannose receptor can target antigen to specific cell types.

DC-SIGN (human DC–specific intercellular adhesion molecule-grabbing nonintegrin) is a C-type lectin with a type II membrane orientation. It is a receptor for a number of different pathogens including Ebola virus (Alvarez et al., 2002), cytomegalovirus (Halary et al., 2002), hepatitis C virus (Pohlmann et al., 2003), human immunodeficiency virus-1 (Geijtenbeek et al., 2000) Dengue virus (Tassaneetrithep et al., 2003), *Helicobacter pylori* and certain strains of *K. pneumoniae* (Appelmelk et al., 2003). How one receptor can

bind so many different pathogens is not clear. The recognition of these pathogens is probably mediated by its CRD that has specificity for carbohydrate residues such as Lewis-X, D-Man and L-Fuc. It was shown that DC-SIGN interacted with *Helicobacter pylori* and *K. pneumoniae* through LPS structures containing Lewis-X or mannose, respectively (Appelmelk et al., 2003). The mannose-capped cell-wall component of *Mycobacteria tuberculosis,* lipoarabinomannan, also interacts with DC-SIGN (Maeda et al., 2003). Recent studies have shown that mycobacteria can suppress DC function by binding to DC-SIGN and modulate the immune response by shifting the Th1 and Th2 cell balance (Geijtenbeek et al., 2003). This highlights the possible exploitation of DC-SIGN by pathogens to induce an inappropriate immune response or to gain entry into a cell to replicate. In contrast to these findings DC-SIGN may be important in the capture and internalisation of pathogens for processing and antigen presentation (Engering et al., 2002).

SIGN-R1 is one of five recently identified mouse genes that share amino acid sequence homology to human DC-SIGN. SIGN-R1 is not expressed on DCs. It is expressed at high levels by medullary and subcapsular sinus macrophages in lymph nodes and by marginal zone macrophages in the spleen (Kang et al., 2003). SIGN-R1 has been shown to function *in vivo* as a PRR mediating the uptake of CPS of *S. pneumoniae* in the marginal zone of the spleen (Kang et al., 2004).

C: Toll-Like receptors.

TLRs have been identified as a major class of signalling receptors in innate immunity (Takeda et al., 2003). The TLR family now consists of 10 members (TLR1-TLR10). The cytoplasmic portion of TLRs shows a high similarity to that of the IL-1 receptor family, and is called the Toll/IL-1 receptors (TIR) domain. In spite of this similarity, the extracellular portions of both receptors are structurally unrelated. IL-1 receptors possess an Ig-like domain, whereas TLRs have leucine-rich repeats in the extracellular domain (Takeda and Akira, 2003). These receptor families function through the same signalling molecules, including MyD88, IL-1 receptor associated kinase (IRAK), TNF receptor associated factor (TRAF)-6, mitogen-activated protein (MAP) kinases and nuclear factor (NF)-κB (Akira, 2003). Activation of TLRs results in the activation of an inflammatory response. Individual TLRs recognise distinct structural components of pathogens and some studies also suggest recognition of endogenous ligands. These will be discussed in the following sections. The TLR expression varies with cell type. Human blood $CD11c^+$ DCs do not express TLR9, but express all other TLRs, whereas human plasmacytoid DCs (PDC) do not express TLR2, 3, 4, 5 and 8 but express the others. Murine splenic $CD4^+$, $CD8\alpha^+$, and $CD4^-$ $CD8^-$ DCs and also PDCs express all TLRs, with one exception. The $CD8\alpha^+$ subset which does not express TLR7 (Edwards et al., 2003). Mouse monocytes and macrophages express mRNA for most of the TLRs except TLR3 (Muzio et al., 2000). Human B cells have been shown to express TLR1, 6, 7, 9 and 10 (Hornung et al., 2002). Other cell types such as neutrophils (Hayashi et al., 2003) and

mast cells (Supajatura et al., 2001) express a range of TLRs. Why the TLR repertoire varies amongst cell types is still not clear.

TLR1, TLR2 and TLR6

TLR2 recognises a variety of microbial components. These include lipoproteins from a variety of pathogens, LTA from Gram positive bacteria, lipoarabinomannan from mycobacteria, a phenol-soluble modulin from *Staphylococcus epidermidis,* porins from *Neisseria* and atypical LPS from *Porphyromonas gingivalis* (Takeda et al., 2003). The importance of TLR2 in the recognition of peptidoglycan has been shown in mice that are deficient in TLR2 (Takeuchi et al., 2000). The mechanism by which TLR2 recognises many structurally different microbial components may be due to its association with TLR1 and TLR6.

TLR2 has been shown to interact physically with TLR6 in the cell (Ozinsky et al., 2000). Studies in TLR6-deficient mice have shown that TLR6 cooperates functionally with TLR2 to recognise microbial lipopeptides and thus able to distinguish between pathogens (Takeuchi et al., 2001). Bacterial lipopeptides have an amino-terminal cysteine residue that is triacylated in contrast to mycoplasmal macrophage-activating lipopeptide 2 (MALP-2) which is diacylated. Both TLR2 and TLR6 are required to recognise MALP-2, whereas bacterial lipopeptides only require TLR2. TLR6 therefore associates with TLR2 to recognise subtle differences between triacyl and diacyl lipopeptides. TLR1 has also been shown to associate with TLR2. TLR1 and TLR2 have

been shown to confer responsiveness to various soluble factors of *N. meningitidis* (Wyllie et al., 2000).

TLR3

TLR3 has been shown to recognise double stranded RNA (Alexopoulou et al., 2001). Double stranded RNA is an intermediate produced by some viruses during their replication cycle. Double stranded RNA has been shown to induce the synthesis of type 1 interferons (IFNα/β), which have antiviral activities. Similar observations have been made with synthetic double stranded RNA, polyinosinic-polycytidylic acid (poly(I:C)). Mice deficient in TLR3 fail to respond to both viral double stranded RNA and poly(I:C) (Alexopoulou et al., 2001).

TLR4

TLR4 is an essential receptor that transduces the signals of LPS. This was demonstrated by the discovery that C3H/HeJ mice (hyporesponsive to LPS) have a single point mutation in a conserved region of the cytoplasmic domain of TLR4. In addition, mice deficient in TLR4 had similar manifestations to C3H/HeJ mice (Hoshino et al., 1999). The recognition of LPS by TLR4 requires several additional molecules. LPS binds to LPS-binding protein (LBP) in serum and the LPS-LBP complex is associated with CD14 present on the surface of mononuclear phagocytes. Mice deficient in either LBP and CD14 have abrogated responses to LPS (Haziot et al., 1996, Jack et al., 1997). Another molecule involved in the LPS response is MD-2, a protein that associates with the extracellular portion of TLR4. Macrophages, DCs and B

cells from MD-2 deficient mice fail to respond to LPS (Nagai et al., 2002). An additional component involved in the recognition of LPS in B cells is RP105. RP105 contains a leucine-rich repeat domain that is structurally related to those found in the extracellular portion of the TLRs and is preferentially expressed on B cells (Miyake et al., 1995). A functional association of RP105 and TLR4 has been shown. Thus, RP105 together with TLR4 are involved in the recognition of LPS in B cells.

In addition to LPS, TLR4 recognises several other ligands. Taxol, a diterpene purified from the bark of the Western Yew, has been shown to have LPS-like activities in mice (Byrd-Leifer et al., 2001). TLR4 has also been shown to recognise various endogenous ligands, for example heat shock protein 60 (HSP60) (Ohashi et al., 2000), HSP70 (Asea et al., 2002), type III repeat extra domain A of fibronectin (Okamura et al., 2001), oligosaccharides of hyaluronic acid (Termeer et al., 2002) and heparan sulphate (Johnson et al., 2002). These host products are often found during injury, infection and autoimmunity, thus TLR4 may be important in the recognition of endogenous ligands involved in the inflammatory response. However, it is also possible that these host products may contain LPS which could activate TLR4.

Surprisingly, there is no evidence to show direct binding of LPS to TLR4. Triantafilou proposed a model of LPS recognition by a cluster of receptors associated with lipid rafts (Triantafilou and Triantafilou, 2002). CD14, HSP70, HSP90, are constitutively found on lipid rafts whereas chemokine receptor 4 (CXCR4), growth differentiation factor 5 (GDF5) and TLR4 are all

found in lipid rafts only after LPS stimulation. LPS therefore generates an association of an array of receptors that could be involved with LPS recognition.

TLR5

TLR-5 has been shown to be activated by bacterial flagellin (Hayashi et al., 2001). Flagellin is a monomeric constituent of bacterial flagella, which are polymeric rod-like appendages extending from the outer membrane of bacteria. Flagella serve as the propellers that move bacteria through an aqueous environment.

TLR7, TLR8 and TLR10

TLR7 recognises synthetic imidazoquinolines (Hemmi et al., 2002). Imiquimod and R-848 are examples of imidazoquinolines that possess potent antiviral and anti-tumour properties. They induce the production of inflammatory cytokines, such as IFN-γ. Both TLR8 and TLR7 have been shown to mediate independently recognition of R-848 in humans (Jurk et al., 2002). These compounds have structures similar to nucleic acids. Two recent papers have described the recognition of single stranded RNA by TLR7 and TLR8. Heil and colleagues have shown that mouse TLR7 and human TLR8 recognises guanosine and uridine rich single stranded RNA oligonucleotides derived from HIV-1 (Heil et al., 2004) and Diebold and colleagues demonstrated endosomal recognition of influenza genomic RNA and signalling by TLR7 (Diebold et al., 2004). This is the first time

physiological ligands for TLR7 and TLR8 have been shown. Very little is known about TLR10 and no ligands have yet been published.

TLR9

An essential role for TLR9 in the recognition of bacterial CpG DNA, a potent activator of immune cells, has been shown. Mice deficient in TLR9 fail to produce a response to CpG DNA (Hemmi et al., 2000). Bacterial DNA contains unmethylated CpG motifs, which confer the immunostimulatory activity. In vertebrates, the frequency of CpG motifs is very much decreased and the cytosine residues of the CpG motif are highly methylated, which results in the abrogation of the stimulatory activity. Various synthetic oligodinucleotides containing CpG motifs can also activate various immune cells. CpG DNA has been shown to activate DCs to produce a Th1-like immune response (Wagner, 1999) and is a strong inducer of B cells activation (Krieg et al., 1995), therefore CpG DNA has promising potential as an adjuvant.

Although most studies have focused on bacterial DNA, it has also been shown that TLR9 may be important in the recognition on viral DNA. DNA viruses contain abundant CpG motifs (Lundberg et al., 2003). TLR9-dependent recognition has been shown for Murine Cytomegalovirus (Krug et al., 2004) and Herpes Simplex virus type 1 (Hochrein et al., 2004).

D: Complement Receptors

Complement proteins in the serum can opsonize microbes through antibody-independent and antibody-dependent mechanisms as described in Section 1.3.2a on complement. Microbes and microbial components that are opsonized are recognised and internalised *via* specific complement receptors. Phagocytic complement receptors include complement receptor 1 (CR1 or CD35), complement receptor 3 (CR3, CD11b/CD18 or Mac-1), and complement receptor 4 (CR4 or CD11c/CD18) (Underhill and Ozinsky, 2002). Complement receptor 2 (CR2 or CD21) has not been shown to be a phagocytic receptor.

C3, when activated on a cell surface becomes covalently bound as C3b which is subsequently cleaved to yield iC3b. There is evidence to suggest that CR3 and CR4 are involved in the phagocytosis of targets opsonised with C3b and iC3b fragments (Cabanas et al., 1999; Ehlers, 2000). Although CR3 is abundantly expressed by circulating monocytes, neutrophils, and NK cells, CR4 is the most abundant C3 receptor on tissue macrophages (Myones et al., 1988). Interestingly, in mouse, CR4 is highly expressed on dendritic cells but its role is poorly defined. The diverse functions and activites of CR3 have highlighted its key role in innate immunity. Some studies have suggested that CR3 can bind some microbial molecules directly (Cywes et al., 1997; Thornton et al., 1996) although this remains controversial. CR3 is not only important for phagocytosis but also leukocyte adherence and migration. Neutrophils from CR3-deficient mice have impaired adhesion and

phagocytosis (Lu et al., 1997). These mice are also defective in bacterial clearance (Rosenkranz et al., 1998).

Human CD35 and CD21 are products of separate genes and are widely expressed, whereas murine CD35 and CD21 are alternatively spliced products of a single gene as designated Cr2 and are expressed almost exclusively on B cells and follicular dendritic cells (FDC). Human CD35 has been shown to bind MBL (Ghiran et al., 2000) and C1q (Klickstein et al., 1997) as well as C3b and C4b opsonized antigens (Fearon, 1980). Human CD21 is a receptor for C3d. It can function as a member of a signalling complex CD21/CD19/CD81 which is important in B cell activation. Murine CD35 binds C3b, iC3b, C3d C3dg, and C4b (Pramoonjago et al., 1993), whereas human CD35 does not bind iC3b, C3dg or C3d (Cooper, 1969; Fearon, 1980). Murine CD21 binds the same ligands except for C3b and C4b. Like human CD21, murine CD21 is expressed as a complex with CD81 and CD19. Both murine CD35 and CD21 are important in humoral responses. Binding of C3d to antigen targets antigen to B cells and FDCs. The role of these receptors in adaptive immunity is described in a later section.

1.3.3 Natural antibodies and B-1 cells.

Natural antibodies are an important part of innate immunity in the recognition of microbial carbohydrates. Natural antibodies are mostly of the IgM isotype and can bind to certain antigens or pathogens, even if the host has never been exposed to them previously. Natural IgM binds to invading pathogens as they enter the host resulting in complement activation as a first line of

defence. Large proportions of these natural antibodies are polyreactive to conserved structures, such as nucleic acids, heat shock proteins, carbohydrates, phosphatidylcholine and phospholipids (Kantor and Herzenberg, 1993; Hardy and Hayakawa, 1994). These natural antibodies appear in the absence of apparent antigenic stimulation, and are secreted by the long-lived self renewing B1 subset of B cells (Boes. 2000).

B1 cells, although constituting only a minor fraction of B cells in the spleen and lymph nodes of mice, represent the main B cell population in the peritoneal and pleural cavities. B1 cells differ from B-2 cells (conventional B cells) by their differentiation during fetal and neonatal development. Early in ontogeny, TdT (terminal deoxynucleotidyl transferase) expression is minimal (Hayakawa and Hardy, 2000). This lack of N region insertions results in the predominant development of B cells expressing germ-line encoded antibody specificities. These germline encoded antibodies have evolved to protect against pathogens, recognising common motifs of bacteria. There also appears to be a lack of somatic mutation of V regions of the antibody genes, therefore natural antibodies tend to be of low affinity. Compared to B-2 cells, B-1 cells have a much-restricted antibody repertoire.

B-1 cells are the source for much of the spontaneously produced natural antibodies, but they also participate in a variety of immune responses. It has been suggested that B-1 cells together with marginal zone B cells are important in the response against microbial carbohydrates.

1.4 Humoral response to T-independent type 2 antigens

1.4.1 T-independent and T-dependent antigens

With regard to their capacity for antibody induction, antigens can be classified as either T lymphocyte-dependent (TD) or as T lymphocyte-independent (TI) (Mond et al., 1995a). TD antigens, such as proteins and peptides, require T cell help for B cell activation and antibody production. This involves the binding of protein to an antigen-specific B cell receptor (BCR) followed by cognate interaction with T cells expressing T cell receptors (TCR) that recognise antigen-derived peptides presented by class II major histocompatibility complexes (MHC II) on the surface of the B cell. TI antigens do not require T cell help for B cell activation; these antigens can induce antibody responses in thymectomised mice (Davies et al., 1970). TI antigens are sub-divided into type 1 (TI-1) and type 2 (TI-2), based on their ability to induce antibody responses in CBA/N (X-linked immunodeficiency) and neonatal mice (Amsbaugh et al., 1972). Both CBA/N and neonatal mice respond normally to TD and TI-1 antigens but fail to produce an antibody response to TI-2 antigens. The cause of the immunodeficiency in the CBA/N mice has been identified as a point mutation in the gene encoding Bruton's tyrosine kinase (Btk) (Thomas et al., 1993). Btk participates in signalling pathways downstream from the B cell receptor (BCR), which appear to be critical for survival and differentiation of activated B cells in response to polysaccharide antigens (Desiderio., 1997). For TD antigens CD40 co-stimulation occurs as well as BCR signalling. CD40 signalling converges with the BCR signalling pathway, which may explain why the defect in antibody

production after BCR ligation of Btk deficient B cells can be overcome in TD responses (Khan et al., 1997).

TI-1 antigens are polyclonal B cell activators/mitogens that can activate B cells regardless of specificity. LPS is an example of this type of antigen. LPS is thought to induce activation of B cells through either TLR4 or RP105, this results in B cell proliferation and secretion of large amounts of antibody (Hoshino et al., 1999; Ogata et al., 2000). LPS can also activate B cells by cross-linking of the BCR with O-antigen sidechain, although this response is more likely to be classified as TI-2 (Nishiuchi et al., 2000).

TI-2 antigens are antigens that consist of repetitive biochemical structures, such as polymeric protein antigens, trinitrophenyl-Ficoll (TNP-Ficoll) and dinitrophenyl-Ficoll (DNP-Ficoll). A clinically important group among the TI-2 antigens are the bacterial CPSs (Rijkers and Mosier, 1985). The main characteristics of these antigens are that they have late onset of responsiveness, infants under the age of 2 years being unable to make antibody responses to most polysaccharides, restricted antibody switching and a lack of affinity maturation and memory function (Mond et al., 1995b).

1.4.2 B cell activation by TI-2 antigens

The two-signal hypothesis of antibody production to TI-2 antigens was first proposed by Vos and colleagues (Vos et al., 2000, Mond et al., 1995b). The first signal involves the cross-linking of the BCR by multivalent TI-2 antigens. Structural analysis of TI-2 antigens showed that they have a minimal

molecular weight of 100kDa (Dintzis et al., 1976). The cross-linking of membrane BCRs by TI-2 antigens is critical for B cell stimulation. It was estimated that B cell activation requires cross-linking of a minimum of 10-20 BCRs (Dintzis et al., 1982). The second signal can be provided through non-antigen-specific stimuli and receptors. The second signal can either directly target B cells or have indirect effects, such as induction of cytokines or upregulation of costimulatory molecules by other cells of the immune system. These second signals can induce class switching, antibody secretion or B cell proliferation. The second signal is also an important requirement for preventing activation of B cells specific to other multivalent self antigens such as DNA, collagen, actin and tubulin (Mond et al., 1995a). Possible second signals are discussed in the following sections.

1.4.3 Modulation of TI-2 responses by complement

In addition to its importance in innate immunity complement is also involved in adaptive responses. Complement provides an essential link between the adaptive and innate immune system by enhancing uptake of antigen in specialised microenvironments and by augmenting the BCR cross-linking signal (Carroll, 1998).

TI-2 antigens can activate complement by either the alternative or the classical pathway. If there is specific antibody present, complement will be activated via immune complexes. In the absence of specific antibody, covalent attachment of C3b (and subsequent proteolytic fragments iC3b and C3d) may occur by activation of the alternative or MBL pathway. Purified pneumococcal polysaccharides can induce direct activation of C3 via the

alternative pathway (Griffioen et al., 1991), as was also found to be the case for Ficoll (van den Eertwegh et al., 1992).

In the case of both TD and TI antigen responses, two mechanisms can account for actions of complement. Firstly, complement fragments bound to TI-2 antigens allow the engagement of complement receptor CD21 on the surface of B cells. CD21 is part of a B cell surface complex that includes CD19 and CD81. Co-ligation of the BCR and the complement complex (CD19/CD81/CD21) by C3 fragments bound to antigen results in a lowering of the threshold for B cell activation. Secondly, the presence of complement receptors, CD21 and CD35, on the surface of FDCs also acts to retain the antigen-complement complex.

Various studies have shown complement and complement receptors to be important in T-independent antibody responses. The conjugation of C3d to a pneumococcal CPS resulted in an increased antibody response and class switching from predominantly IgM to IgG1 in mice compared to mice immunised with pneumococcal CPS alone (Test et al., 2001). Marques and colleagues also showed a correlation between the amount of C3d deposited on pneumococcal polysaccharides and immunogenicity (Marques et al., 1992). Mice deficient in either CD21 and CD35 (Cr2-/-) and complement component C3 had a reduced IgM and IgG response to group B Streptococcus type III CPS (Pozdnyakova et al., 2003). No IgG response to DNP-Ficoll was observed in Cr2-/- mice (Haas et al., 2002). MZ B cells, which are of central importance in TI-2 responses (see section 1.4.7),

express high levels of CD21 when they reach maturity at about 18 months in humans. Maturation of these cells correlates with responsiveness to TI-2 antigens. These studies illustrate the importance of complement in modulating TI-2 responses by increasing immunogenicity or by providing signals to allow immunoglobulin class switching.

1.4.4 Modulation of TI-2 responses by T cells

The finding that certain antigens could stimulate antibody responses in athymic mice, which led to their classification as T independent, suggested that T cells played little if any role in the immune response to these antigens. It is now clear that T cells can regulate responses to TI antigens, but not *via* classical cognate, MHC class II-restricted help. Mice deficient in MHC class II respond normally to TNP-ficoll but TD antibody responses are abolished (Markowitz et al., 1993). The possibility of other costimulatory interactions between T and B lymphocytes is discussed in the following paragraphs.

The production of TD antibody responses is critically dependent on the interaction of CD40 on B cells and CD40L on CD4+ T cells. CD40 interaction with CD40L plays an important role in antibody class switching (Warren and Berton, 1995) and is essential for germinal centre formation and memory function (van Kooten and Banchereau, 1997). The role of CD40L-CD40 interaction in TI-2 responses remains unclear. Renshaw and colleagues showed that CD40L-deficient mice responded normally to TNP-ficoll and DNP-ficoll suggesting TI-2 antibody responses are CD40L-independent (Renshaw et al., 1994). However other studies with pneumococcal CPSs

have shown that administration of anti-CD40 can enhance anti-polysaccharide responses (Dullforce et al., 1998) and the immunisation of an antagonist to CD40L in mice resulted in the abrogation of the anti-polysaccharide response (Jeurissen et al., 2002). Further to this, CD4+ T cells were shown to be important in the enhancement of antibody responses to pneumococcal CPSs and this could be prevented by administration of a CD40L antagonist. Evidence thus suggests that T cells can contribute to the regulation of anti-pneumococcal CPS responses in a CD40L-dependent manner.

CD28, expressed constitutively on T cells, mediates a positive signal through binding to CD80 and CD86 on B cells. CTLA-4, a close relative of CD28, also binds to CD80 and CD86 but acts as a negative regulator of T cell activation. The role of CD28 and CTLA-4 in the regulation of TI-2 antigens has only been the subject of a few studies. Anti-CTLA-4 antibody treatment resulted in the enhancement of isotype switching in polysaccharide-specific antibody responses (Jeurissen et al., 2004). This suggests that CTLA-4 can down regulate antibody responses to TI-2 antigens. In the case of CD28, CD28 knockout mice responded poorly to phosphorylcholine in comparison to wild type mice, suggesting that CD28 may positively regulate TI-2 antibody responses (Wu et al., 2000).

The B cell lymphocyte stimulator (BlyS) is expressed on macrophages, monocytes, DCs and T cells, and has three known receptors (TACI, BCMA, and BAFF-R) which are expressed on B cells. APRIL, a close relative of

BlyS, is expressed on activated T cells and can interact with TACI and BCMA but not BAFF-R. TACI knockout mice were shown to have a reduced antibody response to NP-ficoll compared to wild type mice (von Bulow et al., 2001). BAFF-R deficient mice responded normally to TI-2 antigens (Miller et al., 1992). These studies suggest that the interaction of BlyS and APRIL with TACI, but not BAFF-R, play a role in TI-2 immune responses.

CD1-restricted T cells have also been implicated as a possible T cell subset that helps mount an antibody response to polysaccharide antigens. The CD1 proteins are antigen-presenting molecules that present microbial nonpeptide antigens, such as lipids and glycolipids to T cells. While humans express CD1a, b, c, d, and e, mice only express CD1d (Brigl and Brenner, 2004). Similar in structure to MHC class I, the CD1 heavy chain associates with β2 microglobulin to form a heterodimer that is expressed on the cell surface of APCs (Porcelli et al., 1998), mice that do not express β2 microglobulin have a reduced responses to DNP-Ficoll (Christianson et al., 1997). Interestingly, CD1d expression is high on splenic marginal zone B cells in mice, which is a cell type suggested to be important in the generation of antibodies to TI-2 antigens (Roark et al., 1998). Some CPS from encapsulated bacteria are naturally lipidated (Arakere et al., 1994). Fairhurst and colleagues demonstrated that CD1-resticted T cells proliferated in response to a nonpeptide antigen from *H. influenzae* which they suggest may be lipidated CPS (Fairhurst et al., 1998a). It is hypothesised that B cells internalise the lipid anchored CPS through surface IgM, process the antigen, and present the lipid fragment in context with CD1. In this manner, B cells directly elicit T-

cell help in their production of anti-polysaccharide antibodies (Fairhurst et al., 1998b).

The direct interaction of B and T cells is not the only possible mechanism of T cell help for TI-2 antibody responses. T cells produce cytokines that could provide signals to B cells. Van den Eertwegh and colleagues showed that when murine spleen cells are stimulated with a TI-2 antigen, T cells produce IL-2, IL-4 and IFNγ (Van den Eertwegh et al., 1993). Other cells of the immune system have also been implicated in cytokine production to provide a second signal for TI-2 responses. These are discussed later.

1.4.5 Modulation of TI-2 responses by NK cells

NK cells are a population of non-T cells that can mediate cytotoxicity and produce a panel of cytokines (Biron et al., 1999). It is possible that NK cells could replace or synergise with T cells in providing ancillary help for TI-2 antibody responses. One of the ways in which NK cells may regulate immune responses is through cytokine secretion. NK cells have been reported to secrete various cytokines such as TNF-α and IFN-γ (Biron et al., 1999). IFN-γ has been shown to be important for class switching and Ig secretion and it is therefore possible that NK cells could provide IFN-γ for limited class switching events in TI-2 responses. *In vitro* studies have suggested NK cells play an important role in TI-2 antibody responses, through the induction of IFN-γ (Vos et al., 1999; Snapper et al., 1994). A number of studies have also been carried out *in vivo*. Szomolanyi-Tsuda *et al* showed that antiviral TI-2 antibody responses could be induced in NK-deficient mice, but the presence

of NK cells was required to promote isotype switching (Szomolanyi-Tsuda et al., 2001). However, another study carried out by Kim *et al* showed that there was no difference in the antibody response or isotype switching to TNP-ficoll in NK-deficient mice compared to wild type (Kim et al., 2000). It is therefore not clear whether NK cells are essential for the regulation of TI-2 responses. If there is a major contribution through cytokine release it is possible that the antibody response in NK-deficient mice is normal because of the production of cytokines from other cell types.

1.4.6 Modulation of TI-2 responses by DCs and macrophages.

The link that TLRs provide between the innate and the adaptive immune system suggests they have a possible role in the regulation of B cell responses. DCs can be stimulated via their TLRs by intact bacteria and bacterial wall components to secrete the B-cell stimulatory cytokines IL-1 and IL-6, and to induce NK cell production of IFNγ via IL-12 (Reis e Sousa et al., 1997). Bacterial DNA has also been shown to induce DC maturation and cytokine secretion (Sparwasser et al., 1998). Stimulation of macrophages with microbial stimuli can also result in their activation and the release of cytokines (Stacey et al., 1996; Yamamoto et al., 1997; Zhang et al., 1999). The TLR system can therefore provide B cells with second signals that are based on non-self patterns (Medzhitov and Janeway, 1997). Snapper and colleagues showed that the combination of bacterial lipoproteins and a multivalent antigen resulted in enhanced B cell proliferation and antibody production relative to the multivalent antigen alone (Snapper et al., 1995). A more recent study by Kovarik *et al* demonstrated an increase in antibody response to TNP-ficoll when mice were immunised with TNP-ficoll and CpG

oligodeoxynucleotides compared to TNP-ficoll alone. Interestingly, CpG oligonucleotides failed to enhance the antibody response to pneumococcal CPSs (Kovarik et al., 2001). These studies show that TLR ligands can provide help for some TI-2 antibody responses. As mentioned in previous section BlyS which is expressed on DCs and macrophages is also important in the modulation of TI-2 responses.

1.4.7 Lymphoid tissues and cell types involved in TI-2 responses

Lymphoid organs are organised tissues containing large numbers of lymphocytes in a framework of non lymphoid cells. Lymphoid organs can be divided broadly into primary lymphoid organs, where lymphocytes are generated, and secondary lymphoid organs, where adaptive responses are initiated and lymphocytes are maintained. Bone marrow and thymus are both primary lymphoid organs whereas lymph nodes and spleen are secondary lymphoid organs.

In contrast to TD responses, which occur in all secondary lymphoid organs, the spleen has a far greater capacity to respond to TI-2 antigens than lymph nodes. A functional spleen is therefore essential for TI-2 responses (Amlot et al., 1985). It has been shown that the absence or dysfunction of the spleen results in increased risk of infections from encapsulated bacteria. Splenectomised patients show an increased risk of developing severe bacterial infections (Krivit., 1977).

The spleen contains both white and red pulp. The white pulp consists of three distinct compartments; periarteriolar lymphocyte sheath (PALS), follicles and the marginal zone. The PALS is preferentially a T cell area, whereas the follicles are B cell areas where the germinal center reaction occurs. The PALS and follicles are surrounded by the marginal zone which forms a junction between the red and white pulp. The marginal zone is where blood leaves the arterial system into the venous sinuses. The architectural structure of the marginal zone results in a strongly reduced blood-flow allowing intimate contact between effector cells and blood borne antigens. Marginal zone contains distinct cell sub-populations, which include marginal zone macrophages (MZM), marginal zone metallophilic macrophages (MZMM), marginal zone dendritic cells (MZDC) and marginal zone B cells (MZ B cells).

The non lymphoid cells of the marginal sinus are very specialised. There are two subpopulations of macrophages, the MZMM and the MZM. MZM are identified by high level expression of sialoadhesin (Crocker and Gordon, 1989), they have close contact with MZ B cells and are situated at the red pulp side of the marginal sinus (Dijkstra et al., 1985). MZM have been shown to selectively retain and uptake ficoll (Dijkstra et al., 1985) and neutral polysaccharides (Kraal et al., 1989), and evidence suggests that the uptake of TI-2 antigens is receptor-mediated. Mouse MZM express a number of receptors including MARCO (Elomaa et al., 1995) and SIGN-R1 (Geijtenbeek et al., 2002). MARCO has been shown to bind a range of microbial antigens including *Staphylococcus aureus* and *Eschericia coli* whereas SIGN-RI is the predominant receptor for uptake of the polysaccharide dextran (Geijtenbeek

et al., 2002; Kang et al., 2003) and *S. pneumoniae* and its CPS (Kang et al., 2004). MZMM can be distinguished from MZM by antibody staining with MOMA-1; they are found at the inner border of the marginal sinus and they fail to take up Ficoll (Kraal and Janse, 1986). Although much of the evidence presented above suggests a role for MZM in the response to TI-2 antigens, depletion of these cells in mice does not alter the response to the model antigen, TNP-Ficoll (Kraal et al., 1989). These results suggest that MZM are not involved in the TI-2 response or a compensation mechanism is used. Similar results were observed in mice that were depleted of MZMM (Buiting et al., 1996).

MZ B cells have been shown to have an essential role in the immune responses to TI-2 antigens. Deficiency of the tyrosine kinase Pyk-2 in mice results in a defect in the MZ cell population. These mice displayed a marked suppression of TI-2 antibody responses (Guinamard et al., 2000). Infant MZ B cells which do not respond to TI-2 antigens have been characterised as $CD21^{low}$. The appearance of adult phenotype of MZ B cells ($CD21^{hi}$) also correlates with the ability to mount an immune response to polysaccharides, including those from encapsulated bacteria (Timens et al., 1989). MZ B cells preferentially secrete antibodies of IgM and IgG3 isotypes and the subset is enriched in B cell receptor specificities for bacterial pathogens such as phosphorylcholine (Oliver et al., 1997), reflecting the role of MZ B cells in the primary TI-2 response. MZ B cells represent 5% of splenic B cells in mice and they have a distinct surface phenotype compared to follicular B cells (MZ B cells are IgM^{hi} $IgD^{low/-}$ $CD21^{hi}$ $CD23^{low/-}$, follicular B cells are IgM^{hi} IgD^{hi}

CD21^{inter} CD23^{hi}). In mice, MZ B cells also express high levels of CD1d (Roark et al., 1998).

After maturation, B cells either recirculate through lymphoid organs as part of a long-lived pool (follicular B or B2 B cells) or join more static compartments. In contrast, the MZ B cells are fixed and resident in the spleen, and are positioned for rapid activation by blood borne antigens thus helping to bridge the time gap between innate and adaptive immune responses. MZ B cells have been shown to respond faster than follicular B cells to antigens. MZ B cells express higher levels of CD80 and CD86 when freshly isolated and following *in vitro* stimulation than follicular B cells (Oliver et al., 1999). MZ B cells are also capable of generating plasma cells more efficiently and quickly than recirculating B cells. This is because recirculating B cells are recruited to germinal centres and go through affinity maturation before differentiation into plasma cells (Martin and Kearney, 2000). MZ B cells are present in nude and thymectomised rats, suggesting that they do not represent postgerminal center T dependent B cells (Kumararatne and MacLennan, 1981). These features suggest that MZ B cells are in a state of partial activation.

1.5 Polysaccharide and conjugate vaccines

H. influenzae, *S. pneumoniae* and *N. meningitidis* are examples of encapsulated bacteria which have a major impact on global disease burden. Antibodies to the CPS of these bacteria have been shown to be protective (Macleod et al., 1945). Many early vaccines consisted solely of purified CPS. The first *N. meningitidis* CPS polysaccharide vaccine was licensed in 1972,

followed by 7- and 23-valent vaccines against *S. pneumoniae* (1977 and 1983 respectively), and *H. influenzae* type b (1985). These vaccines are efficacious in immunocompetent adults (Shapiro et al., 1991), but due to their T-independent type II nature, are not generally effective in children under the age of 2 (Karma et al., 1985). In addition, CPSs do not elicit memory functions, and hence the immune response can not be boosted. It was therefore apparent that alternative vaccines were required for immunising very young children.

The discovery that the conjugation of polysaccharide to an immunogenic protein carrier could induce a polysaccharide response in infants, that was enhanced both in terms of magnitude and duration compared to polysaccharide alone, resulted in the development of conjugate vaccines. The conjugate vaccine against *H. influenzae* type b (Hib) has been very successful. Prior to introduction of the conjugate vaccine there were 30-60 cases of invasive disease per 100,000 children under five years of age in Europe (Lindberg, 1999; Peltola et al., 1977). The Hib vaccine consists of the polysaccharide type b capsule, made of polyribosyl ribitol phosphate, conjugated to a protein carrier. Four different carrier proteins have been used, diphtheria toxoid, tetanus toxoid, CRM197 (non toxic variant of diphtheria toxin), and meningococcal outer membrane protein complex. All four conjugates were licensed in the early 1990's. In the UK the number of cases of invasive disease reduced by 98% after the introduction of the conjugate vaccine (Moxon et al., 1999) and the same was observed in other developed countries. The Hib conjugate vaccine has been shown to have an

efficacy of 90% in developing countries such as The Gambia (Mulholland et al., 1997). The Hib conjugate vaccine has been undoubtedly successful and has paved the way for the development of conjugate vaccines against the meningococcus and pneumococcus. *H. influenzae* disease has the great advantage of being caused predominantly by a single serotype, *H. influenzae* type b. In the case of *N. meningiditis* and *S. pneumoniae* there are many different serotypes which cause disease. Of the 90 identified serotypes of *S. pneumoniae,* 23 serotypes cause invasive disease. A 7-valent conjugate vaccine was licensed in 2000 in the US, although developments are currently underway to produce a vaccine that covers more serotypes. The only concern with this vaccine is the increased incidence of otitis media caused by non-vaccine serotypes (Obaro and Adegbola, 2002; Obaro, 2001). In the case of *N. meningitidis* there are 12 known serotypes. Almost 90% of cases of meningitis are caused by serogroups A, B and C. A conjugate vaccine against serogroup C was licensed in the UK in 2001, and a year later it was shown that carriage of serotype C meningococci was reduced by 66% in adolescents (Maiden and Stuart, 2002). Further studies are underway to produce multivalent meningococcal vaccines. Conjugate vaccines against other infectious pathogens, such as *Staphylococcus aureus* (McKenney et al., 1999), *Streptococcus* Group B (Baker et al., 2001), and *Salmonella typhi* (Szu et al., 1994) are also in development.

Polysaccharide-protein conjugate vaccines convert the immune response from T-independent type II to a T-dependent response (see section 1.4). Polysaccharide vaccines fail to activate helper T cells. They activate B cells

in a T cell independent manner resulting in little or no affinity maturation, class switching, memory, or antibody response in infants. One way to involve helper T cells is to conjugate polysaccharide to a protein carrier. The polysaccharide-protein conjugate can be internalised by B cells and peptides from the protein can be presented by MHC class II to antigen-specific helper T cells. The involvement of T helper cells is expected to result in class switching, generation of memory B cells and thus a T-dependent response to the polysaccharide.

The development of polysaccharide-protein conjugate has been very successful, as shown by the *H. influenzae* type b conjugate vaccine. The conversion of a T independent response to a T dependent response allows the development of memory and responsiveness in infants, a group most at risk from disease due to encapsulated bacteria. Alternative methods of producing a vaccine that can stimulate T-dependent responses are also under study, such as the development of peptides that mimic the immunological properties of polysaccharides.

1.6 Adjuvants

Vaccines come in several forms: live-attenuated, replicating and non-replicating pathogens, inactivated pathogens or their subunits. The last category is the safest, but can lack immunogenicity and often requires adjuvants to elicit an adequate immune response. Adjuvants are defined as a group of heterogeneous compounds, used to evoke or increase an immune response to an antigen (Gupta et al., 1993). Adjuvants have the capability to influence or control many parameters of the immune response such as

antibody production (specificity, titre, memory, duration, class, isotype and avidity) and cell mediated immunity (CD4 or CD8). Adjuvants are thought to act through various mechanisms including induction of increased antigen transportation and presentation by APCs, sustained presence of antigen at injection site to increase antigen exposure time, and induction of cytokine release and upregulation of costimulatory molecules on APCs.

The adjuvants used in human vaccines must fulfil stringent requirements. The adjuvant must be non-toxic, stimulate a strong humoral or cell-mediated immune response, provide long term immunological memory, non-mutagenic, non-pyrogenic, and must be stable at a range of temperatures and pH. Aluminium-based adjuvants are the only ones approved for human use. They are emulsion-based and insoluble, the antigen and adjuvant are sequestered at the injection site and are released over a period of time to stimulate APCs. Aluminium-based adjuvants typically are effective in enhancing antibody responses to protein antigens such as tetanus and diphtheria toxoids. They induce Th2 type responses. They are not effective for cell-mediated immunity therefore aluminium adjuvants are not efficacious for all vaccines. There is therefore a major interest in finding adjuvants that are capable of inducing strong cellular immune responses of a Th1 type.

The activation of the innate immune system results in the initiation of a proinflammatory response, and the eventual activation of the adaptive immune response. Therefore activators of innate immunity can be used as adjuvants. These adjuvants include ligands of TLRs. TLRs induce signaling

pathways, leading to activation of NF-κB in APCs, which results in the expression of cytokine genes, production of costimulatory ligands CD80 and CD86, and activation of adaptive immunity. Unmethylated CpG >2 nucleotides, TLR9 ligand, stimulate immune cells (T cells, B cells, NK cells and macrophages) to produce proinflammatory cytokines, including IL-1, IL-6, IL-12, IL-18, TNF-α and IFN-γ that promote Th1 immunity (Wagner, 1999). LPS also stimulates the production of proinflammatory molecules, although the lipid A part of the molecule is responsible for these properties. Lipid A is extremely toxic so lipid A mimetics have been studied for adjuvant properties. These microbial structures amongst others have an important future in the development of adjuvants for new vaccines.

1.7 Aims

The aim of this thesis is to understand further the interactions of CPS with the immune system and how this can determine or influence immunogenicity and adjuvanticity. The insights provided by this project into the induction of anti-CPS immune responses and possible adjuvant properties may contribute to the improvement of current CPS-based vaccines and adjuvants for vaccine formulations. The specific aims of this study are described below.

Immunomodulatory properties of *K. pneumoniae* CPSs

A number of studies have described possible immunomodulatory properties of *K. pneumoniae* CPS although it is not clear whether these properties can be attributed to the CPS itself or possible contaminants such as LPS or

bacterial DNA. This study is to determine whether CPSs from *K. pneumoniae* have immunostimulatory properties.

- Establish a method of purification of CPS from *K. pneumoniae*.
- Investigate the possible adjuvant properties of purified CPS from *K. pneumoniae.*
- Examine possible mechanisms of adjuvant activity of CPS from *K. pneumoniae*

Role of complement in the humoral response to *S. pneumoniae* CPSs

The role of complement in the humoral response to physiological T-independent type 2 antigens, such as CPS, remains unclear. Many studies have focused on model TI-2 antigens, such as DNP-Ficoll. CPSs from *S. pneumoniae* are commercially available as a 23-valent vaccine and are structurally diverse. This provided an opportunity to study structure-immunogenicity relationship using a relatively large set of CPS with differing structures and establish the role of complement in anti-CPS antibody responses.

- Establish the humoral response to CPSs from *S. pneumoniae* in wild type and complement receptor (CD21 and CD35) deficient mice.

Chapter Two

Materials & Methods

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Antibodies

2.1.1a Monoclonal antibodies used in flow cytometric analysis

Target Molecule	Isotype	Conjugate	Supplier	Catalogue number	dilution
Mouse CD3	Hamster IgG1	PE	PharMingen	553063	1/200
Mouse CD35/CD21	Rat IgG2b	FITC	PharMingen	553818	1/200
Mouse CD45R/B220	Rat IgG2a	APC	PharMingen	553092	1/200

2.1.1b Antibodies used in ELISA assays

Product	Conjugate	Supplier	dilution	Catalogue number
Goat anti-mouse (γ chain specific) IgG	Horseradish peroxidase	Jackson Immunoresearch	1/1000	115-035-164
Goat anti-mouse (γ chain specific) IgG	Alkaline phosphatase	Southern Biotec Associates	1/2000	1030-04
Goat anti-mouse (μ chain specific) IgM	Horseradish peroxidase	Jackson Immunoresearch	1/1000	115-035-0200

Mouse anti-rabbit immunoglobulins	Horseradish peroxidase	Sigma	1/1000	A 2074

2.1.2 Cytometric Bead Array (CBA) kits.

Reagent:	Supplier:	Catalogue number:
Mouse Inflammation CBA (Lot 0000061334)	BD Biosciences Pharmingen	552564
Mouse TH1/TH2 cytokine CBA (Lot 0000049973)	BD Biosciences Pharmingen	551287

2.1.3 Biochemical Reagents

Reagent:	Supplier	Catalogue number:
ABTS (2,2 Azino –bis(3-ethylbenzthiazoline-6-sulfonic acid))	Sigma	A9941
Acetic Acid	BDH	153103D
Acrylamide/ bisacrylamide 30% (v/v) solution	Bio-Rad	161-0156
Alcian Blue	Sigma	A9186
Ammonium hydroxide	Sigma	A6899
Ammonium persulphate	Bio-Rad	161-0700
Betaplate scintillant	Wallac	SC/9200/21
Biuret Reagent	Sigma	690-1
Bovine Serum Albumin	Sigma	A4503

Bromophenol Blue	Sigma	B8026
Carbazole	Sigma	C5132
Carboxyfluorescein diacetate succinimidyl ester (CFSE)	Molecular Probes	V 12883
Chicken gamma globulin (CGG) Lot 53423	Jackson Immunoresearch	003-000-002
Citric acid	Sigma	C0759
Deoxyribonuclease I	Sigma	D5025
Dextran from Leuconostoc ssp. (Mr ~ 40,000)	Fluka	31389
Dextran from Leuconostoc ssp. (Mr ~ 70,000)	Fluka	31390
Dextran from Leuconostoc ssp. (Mr ~ 200,000)	Fluka	31398
Dextran from Leuconostoc ssp. (Mr ~ 500,000)	Fluka	31392
Dextran from Leuconostoc ssp. (Mr ~ 1,800,000)	Fluka	31427
Ethanol	BDH	15338
DNP-Ficoll	Biosearch Technologies	F-1200-10
Ethylenediaminetetraacetic acid (EDTA)	Sigma	E5134
Folin and Ciocalteu's phenol reagent	Sigma	F9252

Formaldehyde (37% solution)	Sigma	F1635
Galacturonic acid	Sigma	48280
Glycerol	Sigma	G6279
Glucuronic acid	Sigma	G5269
Glycine	Sigma	G8898
Hydrogen Peroxide	Sigma	H1009
Ionomycin	Sigma	I0634
Lauryl sulphate	Sigma	L3771
Lipopolysaccharide from *Esherichia coli* O55:B5	Sigma	L2880
Methanol	BDH	10158BG
2-Mercaptoethanol 50mM	Gibco Invitrogen	31350-010
Milk Powder	Marvel	Off shelf
N, N, N', N' –tetra-methyl-ethylenediamine (TEMED)	Bio-Rad	161-0800
(OPD) o-Phenylenediamine Dihydrochloride tablet sets	Sigma	P9187
Periodic Acid	Sigma	P5463
Phenol	Sigma	P5566
Phorbol myristate acetate (PMA)	Sigma	P1585
Phosphate Buffered Saline (PBS), 10x, (0.01M KH_2PO_4, 1.5M NaCl, 0.03M Na_2HPO_4 at pH 7.2), without Ca or Mg.	Gibco Invitrogen	14200-067

p-Nitro-phenyl-phosphate (pNpp)	Sigma	N1891
Polymyxin B sulphate	Sigma	P4932
Potassium hydroxide	Sigma	P5958
Ribonuclease A	Sigma	R4875
Ribonuclease Type XII B	Sigma	R7884
Silver nitrate	Sigma	S6506
Sodium Azide	Sigma	S8032
Sodium Chloride	Sigma	S7653
Sodium Deoxycholate	Sigma	D6750
Sodium Hydroxide	Sigma	S8045
Subtilisin Type VIII	Sigma	P5380
Sucrose	Sigma	S7903
Sulphuric acid	BDH	102761C
Thiomersal	Sigma	T2299
Titermax gold adjuvant liquid	Sigma	T2684
Triton X-114	Sigma	T-114
Trizma base	Sigma	T1503
Tween 20	Sigma	P7949

2.1.4 Tissue Culture Reagents

Reagent:	**Supplier**	**Catalogue number**
Dimethyl sulphoxide (DMSO)	Sigma	D-8418

Foetal bovine serum Lot 40G2027K	Gibco Invitrogen	10270-169
HEPES buffer 1M	Gibco Invitrogen	15630-056
Penicillin/Streptomycin	Gibco Invitrogen	15140-122
RBC lysing buffer	Sigma	R7757
RPMI 1640 with Glutamax 1	Gibco Invitrogen,	61870-010
Trypan Blue	Sigma	T8154

2.1.5 Plastics

Type of Plastic:	**Supplier:**	**Catalogue number:**
15ml round conical tube	Falcon	2096
24-well plates	Falcon	3043
50ml round conical tube	Falcon	2070
96-well immunoplates	NUNC	DIS-971-010P
96-well U bottomed plate	Nunclon	163320
Cell strainer 0.4µm	Falcon	2340

2.2 Methods

2.2.1 Purification of capsular polysaccharides from *Klebsiella pneumoniae.*

2.2.1a Bacterial strains

The following O-antigen and capsular serotype (K) strains of *K. pneumoniae* were obtained from the Statens Serum Institute, Copenhagen, Denmark.

01:K1, O2:K3, 03:K11, O1:K22, O1:K26, O2:K27, O3:K31, 04:K36, R:K40, O1:K46, R:K52 O3:K55, O5:K60.

Bacteria were grown either on nutrient agar or lactose agar plates. For long term storage bacterial strains were aliquoted and stored in Greaves freezing medium (5% BSA, 5% L-glutamic acid sodium salt, 10% glycerol and 80% water) at –80°C. Bacteria were recovered by streaking a partially thawed suspension onto a nutrient agar plate. After incubation overnight at 37°C, colonies were re-streaked and checked for purity.

2.2.1b Growth of bacteria and extraction of capsular polysaccharides

In order to maximise CPS production bacteria were grown on lactose plates. Twenty to fifty plates (9cm diameter) were heavily streaked with bacteria and incubated at 37°C overnight. Bacterial growth was harvested by gently scraping the surface of the agar plates with a spatula. The bacteria were then suspended in 20ml of water and boiled for 10 mins at 100°C to release the CPS. After cooling on ice, cold acetone was added to the preparation so that

the acetone was 80% of the total volume. When a precipitate had formed, the suspension was recovered by centrifugation (Sorvall RT7 plus) for 10 mins at 400 x g at 4°C. The CPS was left to dry at room temperature (RT) overnight then redissolved in PBS. This was followed by centrifugation (Beckman Avanti J.30I) 3 times at 20,000 x g for 30 mins at 4°C. Each time the pellet (consisting of insoluble cell debris and denatured proteins) was discarded. The resulting supernatant containing the CPS was freeze dried (Savant, Freeze drier modulyo), and then redissolved in 5-20ml of water for purification as follows (Cryz et al., 1985).

2.2.1c Purification of capsular polysaccharides

The preparation was subjected to ultra-centrifugation (Beckman Avanti J.30I centrifuge) for 18h at 100,000 x g at 4°C. This was to further remove contaminating protein and LPS from the preparation. The preparation was then digested with Deoxyribonuclease I (70µg/ml) and Ribonuclease A (30µg/ml) or Ribonuclease Type XII B (30µg/ml) in 0.1M Tris/HCl, 4mM $MgCl_2$ in PBS and 0.02% thiomersal for 24h at 37°C in a rocking incubator at an approximate CPS concentration of 500µg/ml as determined by uronic acid assay (Section 2.2.3d). The preparation was further digested with protease [Subtilisin type VIII (70µg/ml)] for 24h at 37°C in a rocking incubator. The preparations were then dialysed [Spectra/por 12-14 kDa MWCO (Spectrum)] extensively against 3L of water for 16h with 3 changes at 4°C. The preparation was then freeze dried (Savant, Freeze drier modulyo).

2.2.1d Gel filtration chromatography – size fractionation and purification

The HPLC system used consisted of a Waters 2690XE separations module, a Waters 2487 dual wavelength UV detector, and a Waters 2410 refractive index detector. System control and data acquisition was performed by Waters Millenium-32 software.

Gel filtration was carried out on a TSK G5000 HPLC column [(7.5mm ID x 30cm) Anachem, Bedfordshire]. The void volume (V_0) was determined by the elution position of blue dextran (2KDa) and the total volume (V_t) was determined by monitoring the elution position of a concentrated salt solution by refractive index. The eluant from samples separated on the column was monitored by refractive index and UV absorbance at 205nm, 260nm and 280nm. The different buffers used are shown in Table 2.1. The flow rate was 0.25ml/min and fractions were collected every 2 mins.

Table 2.1 Gel filtration elution buffers

Buffer	CONDITIONS: Components / temperature / pH
A	PBS **30°C**
B*	(Sodium deoxycholate buffer)10mM Tris-HCl **pH8.7** containing 0.2M NaCl, 1mM EDTA and 0.25% (w/v) sodium deoxycholate at **60°C**
C*	(Sodium deoxycholate buffer)10mM Tris-HCl **pH8.7** containing 0.2M NaCl, 1mM EDTA and 0.25% (w/v) sodium deoxycholate at **30°C**
D*	(Sodium deoxycholate buffer) 10mM Tris-HCl **pH7.5** containing 0.2M NaCl, 1mM EDTA and 0.25% (w/v) sodium deoxycholate at **30°C**
E*	(Sodium deoxycholate buffer) 10mM PBS **pH 7.0** containing 0.25%(w/v) sodium deoxycholate at **60°C.**

***B-E are dissociating buffers designed to separate LPS from CPS.**

Samples typically contained from 100-200µg of CPS in 100-300µl of buffer. For larger scale sample purifications, repeat fractionations of the same CPS were carried out using an automatic injector. Fractions were assayed for carbohydrate using the phenol sulphuric acid assay (Dubois and Giles, 1956). Samples that were eluted with dissociating buffers required the removal of deoxycholate using the method described in Section 2.2.1e before being assayed for carbohydrate. Those containing carbohydrate were pooled and subjected to further analysis.

2.2.1e Removal of sodium deoxycholate dissociating buffer post gel filtration.

The pooled samples that were eluted from the column with dissociating buffers require the removal of deoxycholate before further analysis, as this detergent could interfere with a number of the assays. The pools were placed into dialysis tubing [Spectra/por 12-14 kDa MWCO (spectrum)] and then extensively dialysed against water at RT (deoxycholate precipitates out at 4°C). This removed some of the buffer salts and some but not all of the detergent. The sample was then freeze dried, and then suspended 0.2M NaCl (1-2ml). To remove the remaining detergent cold ethanol was added to 80% of the total volume. The sample was then centrifuged (Sorvall RT7 plus) at 2200 x g for 10 mins. The supernatant was poured off and the pellet was allowed to air dry. The pellet was then dissolved in a minimal amount of water.

2.2.1f Removal of lipopolysaccharide

Extraction with Triton X-114

Extraction of CPS with Triton X-114 has previously been reported to remove LPS from CPS (Adam et al., 1995). The CPS was dissolved in water (0.5mg/ml), Triton X-114 stock solution (11% w/v in water) was added to a final concentration of 2% (w/v) Triton. After cooling on ice, the solution was stirred at 4°C for 30 mins and then incubated at 37°C to induce phase separation. Phases were separated by centrifugation (Sorvall RT7 plus) for 30 mins at 45 x g at 30°C. The lower phase was discarded and Triton X-114 stock solution was added to the upper aqueous phase to a final concentration of 2% (w/v). The procedure was repeated as described above. The aqueous phase was made 0.2M in NaCl and CPS was recovered from the aqueous phase by precipitation with cold 80% (v/v) ethanol. The precipitate was recovered by centrifugation (Beckman Avanti J.301) for 30 mins at 4°C at 20,000 x g and then re-suspended in 0.2M NaCl and the ethanol precipitation was repeated. The sample was dialysed against water. The recovery of the CPS was determined by measuring total carbohydrate by the phenol sulphuric acid assay.

Alkaline de-O-acylation

Alkaline treatment was carried out in order to remove O-acyl fatty acids. This treatment results in the cleavage of the O-acyl fatty acids from the lipid A moiety. Since lipid A is responsible for most of the biological activity of LPS, this treatment detoxifies LPS (Seid et al., 1981). CPS (150µg) was dissolved

in 750µl of 0.1M NaOH. This was incubated overnight at 37°C, and then neutralised with 0.2M acetic acid. CPS was then subjected to dialysis against water.

Gel filtration under dissociating conditions

Using the method already described for gel filtration wherein PBS as an elution buffer, LPS forms high molecular weight micelles and co-elutes with the CPS. However using dissociating conditions it was possible to disrupt micelle formation. Published dissociating conditions using sodium deoxycholate buffer at pH 8.7 and at 60°C (Adam et al., 1995) were used along with modified versions. These are shown in Table 2.1

2.2.2 Purification of lipopolysaccharide from *Klebsiella pneumoniae*

2.2.2a Growth of bacteria and extraction of lipopolysaccharide

Nutrient broth [0.01% bacto-tryptone, 0.005% bacto-yeast extract and 0.01% NaCl (20ml)] was inoculated with one colony to make a starter culture. This was grown overnight at 37°C in a rocking incubator. A large flask containing 2L of nutrient broth was then inoculated with 1ml of the starter culture and grown overnight at 37°C in a shaker incubator at 150 rpm. The culture was centrifuged (Beckman Avanti J301) at 10,000 x g for 30 mins at 4°C. The pellet was re-suspended in 50 – 100ml of PBS and the LPS was extracted with 45% hot aqueous phenol (Westphal and Jann, 1965) as follows. The suspension was heated to 68°C, to this an equal amount of pre-heated 90% (w/v) phenol (saturated aqueous solution) was added. The solution was then

stirred for 15 mins at 68°C. Phase separation was obtained by cooling the solution on ice, followed by centrifugation (Sorvall RT7 plus) at 200 x g for 15 mins at 4°C. The aqueous layer was removed with a pipette, leaving the interface material behind. An equal volume of water to that removed was added, and the phenol extraction was repeated. The two aqueous phases of each extraction were combined and dialysed [Spectra/por 12-14kDa MWCO (Spectrum)] against water at 4°C, with changes of water every 4-6 hours. After dialysis, the preparations were centrifuged (Beckman Avanti J.30I) at 100,000 x g at 4°C overnight. The supernatant was removed and the LPS pellet was re-suspended in water.

2.2.2b Purification of lipopolysaccharide

LPS obtained by aqueous 45% (w/v) phenol extraction was further purified by gel filtration using a HiPrep 26/60 Sephacryl S-300HR column (26 x 600mm), (Amersham Pharmacia Biotech). The buffer used was 20mM Tris-HCL, pH8.5, 2mM EDTA and 1% (w/v) sodium deoxycholate. The flow rate was 0.6ml/min and 5-min fractions were collected. The HPLC system was as described previously. The refractive index of the eluant was monitored together with absorbance at 280nm and 260nm. By this method the LPS, as detected by refractive index, was separated from remaining protein and DNA. LPS-containing fractions were pooled, dialysed [Spectra/por 12-14 kDa MWCO (Spectrum)] at RT against 5L of water with 3 changes, and freeze dried. The LPS was then dissolved in 1-2ml of 0.4M NaCl and precipitated from 80% (v/v) cold ethanol. The precipitate was recovered by centrifugation, left to dry, and dissolved in a small amount of water. The recovery of LPS was determined by the phenol sulphuric acid assay for total carbohydrate.

2.2.3 Analysis and characterisation of capsular polysaccharides from *Klebsiella pneumoniae*

2.2.3a Confirmation of *Klebsiella pneumoniae* capsular polysaccharide serotype

Using rabbit anti-capsular antiserum (Statens Serum Institute, Copenhagen, Denmark) the serotype of the CPS was confirmed by ELISA. The ideal coating concentration could also be confirmed at this time. Immunoplates were coated with 50µl/well of the CPS in a range of different concentrations (10µg/ml – 50µg/ml) diluted in saline. These were incubated overnight in a moist box. They were then washed 3 times with wash buffer [PBS and 0.05% Tween 20]. The antisera were serially diluted 2 fold with PBS and 0.1% Tween 20 and 50µl was added to each well and left for 3h at RT. The plates were then washed with wash buffer. To each well, 50µl of anti-rabbit IgG HRP diluted 1 in 1,000 in PBS and 0.05% Tween 20 was added, followed by incubation at RT for 2h. The plates were then washed and 50µl/well of the substrate 2,2-azino-di-[3-ethylbenthiazoline sulphate] (ABTS) in 50mM citrate-phosphate buffer, pH5.1, was added and allowed to develop. The plates were then read at 405nm on a plate reader [SpectraMAX 340 (Molecular Devices)].

2.2.3b Quantification of carbohydrate

Total carbohydrate content was determined by the phenol sulphuric acid method (Dubois and Giles, 1956). Briefly, 100µl of 5% (w/v) phenol was added to 200ul sample, followed by addition of 1.2ml concentrated sulphuric acid and gentle mixing. Absorbance was measured at 490nm using a spectrophotometer (Beckman DU 640B). Each assay was carried out in duplicate.

2.2.3c Quantification of protein

Protein content was measured by the Folin method (Folin et al., 1927). BSA (10mg/ml) solution was used to make a standard curve for protein content from 10µg to 100µg. The standard sample volume used was 200µl and each standard was done in duplicate. To the sample 2.2ml of Biuret reagent was added. This was mixed and then left at RT for 10 mins. Folin reagent (100µl) was added, sample was mixed and incubated for 30 mins at RT. An absorbance reading was taken at 720nm using a spectrophotometer (Beckman DU 640B).

2.2.3d Quantification of uronic acid

Uronic acid was measured by the carbozole method (Knutson and Jeanes, 1968). All of the *K. pneumoniae* CPS contain one uronic acid residue, either galacturonic acid or glucuronic acid, in each repeating unit of the polysaccharide. By quantifying the amount of uronic acid present in the preparation it is possible to determine the amount of CPS present either on a weight basis or according to the molarity of repeating units. A standard curve was produced using known concentrations of glucuronic acid and galacturonic acid, ranging from 20 to 200nmol. A volume of 350µl of sample (in duplicate) was placed on ice. To this 3ml of cold acid-borate solution [prepared by diluting 2.5ml of stock solution of 4M potassium borate in 1.8M potassium hydroxide to 100ml with concentrated sulphuric acid (final concentration 0.1M borate)] was added. This was mixed and allowed to cool. After cooling 200µl of 0.1% carbazole in absolute ethanol was added, the solution was mixed and then incubated in a water bath at 55°C for 30 mins. Absorbance was measured at 530nm using a spectrophotometer (Beckman DU 640B).

2.2.3e Quantification of lipopolysaccharide

The LPS content of samples was determined by a kinetic turbidometric Limulus amoebocyte lysate (LAL) assay. Reagents for the LAL assay were purchased from Charles River Endosafe, UK and the assay was performed according to manufacturer's instructions with *E.coli* 055 LPS as a standard.

2.2.3f Quantification of nucleic acid

Nucleic acids concentrations were measured spectrophotometrically at 260nm wavelength.

2.2.3g Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis

SDS-PAGE was carried out according to the method of Laemmli (Laemmli, 1970) using BioRad mini protean cell equipment. Samples were prepared by diluting the respective LPS and CPS with 0.25 volumes of sample buffer (0.1M Tris/HCl, pH 8.0, 4mM EDTA, 1M sucrose, 0.1% Bromophenol blue, and 0.2% SDS). Typically 5µg of CPS was loaded into each well, and 0.4µg of LPS was loaded. Samples were mixed thoroughly and then stored at 4°C until required.

A 15% polyacrylamide separating gel was used for the LPS samples and a 10% separating gel was used for CPS samples. Table 2.2 shows the compositions of the 10% and 15% separating gels. Volumes shown are for 10cm x 7.5cm sized gels using 1mm spacers.

Table 2.2 SDS-PAGE separating gel components

COMPONENT	10% separating gel	15% separating gel
Water	5.4ml	3.2ml

1.5M Tris/HCL pH 8.8	3.3ml	3.3ml
30% acrylamide/bisacrylamide (Bio-Rad)	4.4ml	6.6ml
10% SDS	133µl	133µl

Degas under vacuum

10% ammonium persulphate (Bio-Rad)	67µl	46µl
TEMED (Bio-Rad)	7µl	5µl

Mix gel solution thoroughly

The separating gel solution was then poured between two glass plates of a mini-protean cell overlaid with butan-1-ol saturated water and left to polymerise for 1h. The composition of the stacking gel is shown in Table 2.3.

Table 2.3 SDS-PAGE stacking gel components

COMPONENT	STACKING GEL (4.05%)
Water	4.13ml
0.5M Tris/HCl pH 6.8	1.75ml
30% acrylamide/bisacrylamide (Bio-Rad)	0.95ml
10% SDS	67µl

Degas under vacuum

10% ammonium persulphate	32µl
TEMED	7µl

Mix gel solution thoroughly

The butan-1-ol saturated water was poured off the gel. The top of the gel was washed thoroughly with distilled water. The stacking gel solution was then poured on to the separating gel. Combs were carefully inserted into the stacking gel and the gel was left for a further hour to allow for polymerisation.

The samples (5-30µl/well) were then loaded onto the gel. Electrophoresis was carried out using a Power-Pac model 3000 (BioRad). The gels were run at 25mA constant current until the samples had entered the separating gel. At this point the current was increased to 45mA until the tracking dye had reached the bottom of the gel. The gels were ran for approximately 1h. Following electrophoresis the gels were either stained using the silver staining method described in Section 2.2.3i (Tsai and Frasch, 1982) or used for western blotting as described in Section 2.2.3h.

2.2.3h Western blotting

Immediately after electrophoresis, the polyacrylamide gel and a positively charged Nytran membrane (Schleicher & Schuell) and filter paper was soaked for 30 mins in transfer buffer (48mM Tris, 39mM glycine, 20% methanol, and 0.375% SDS). Transfer was achieved by use of a semi-dry transfer cell (Bio-Rad, UK) which was run at 10V for 30 mins. After blotting, the gel was silver stained as described in Section 2.2.3i, and the Nytran

membrane was then blocked with PBS + 3% milk powder for 1-2h. After blocking, blots were incubated overnight at 4°C with a variety of rabbit anti-capsular antisera (Statens Serum Institute), at a dilution of 1/100. This was followed by brief washing with PBS, and four 10-min washes with PBS. The blot was then incubated for 2h with anti-rabbit IgG horseradish peroxidase conjugate at a dilution of 1/800. The membrane was washed as before and immunoblots were developed using 3,3-diaminobenzidine tetrahydrochlorde (DAB) as a substrate. The reaction was stopped with extensive washing with water.

2.2.3i Silver staining

Table 2.4 Silver staining solutions

Solutions	Components
1	40% ethanol / 5% acetic acid made up to 100% with water
2	0.7% periodic acid w/v dissolved in solution 1
3	**Alkaline silver nitrate** Conc. ammonium hydroxide 2ml 0.1M NaOH 28ml 20% silver nitrate 5ml Distilled water 115ml
4	**citric acid – formaldehyde solution** citric acid 50mg 37% formaldehyde 0.5ml made up to 1 litre with water.

Table 2.4 shows components for each solution required for silver staining (Tsai and Frasch, 1982). Immediately after electrophoresis the gel was placed into solution 1 and left overnight to allow for fixation of the gel. It was then placed into solution 2 for 10 mins on a rocking platform. The gel was washed 3 times for 15 mins with distilled water. The gel was then placed into solution 3 for 10 mins. Again the gel was washed 3 times for 15 mins with distilled water before the next solution was added. The gel was added to solution 4, which allows development of the stain. The bands develop over time. To stop development the gel was transferred to water. It is important that all stages are performed on a rocking platform to allow maximum and even coverage of gel with solutions.

The method described above is for staining LPS. For staining CPS 0.01% (w/v) Alcian blue was added to solution 1 prior to silver staining which is necessary to visualise CPS (Min and Cowman, 1986).

2.2.4 Characterisation of humoral response

2.2.4a Protocol of *Streptococcus pneumoniae* CPS immunisations.

Mice

Female or male C57BL/6 (H-2^b), BALB/c (H-2^d) and Cr2 [complement receptors 1 and 2 deficient mice (H-2^b)] mice were obtained from Charles River, UK (Margate, Kent, UK). Cr2 were obtained originally from Dr. M. C. Carroll, Center for Blood Research, Harvard Medical School, Boston, USA.

The animals were kept under specific pathogen free conditions and the mice were used between 8- 12 weeks of age.

Immunisations

Groups of 3 mice were immunised by the intra peritoneal route with a range of pneumococcal CPSs (capsular serotypes 1, 2, 3, 4, 6B, 9N, 9V, 14, 18C, 19A, 19F, 23F) obtained from either the National Institute for Biological Standards and Control (NIBSC, Hertfordshire, UK) or the Statens Serum Institute, Copenhagen, Denmark. These CPSs are constituents of human polysaccharide vaccines for pneumococcal diseases. A range of doses were used (0.1 – 20µg per mouse) in a 500µl volume of sterile PBS. Control mice were given PBS only. The schedule for immunisations and blood sampling is shown in Figure 2.1.

2.2.4b Protocol of *Klebsiella pneumoniae* CPS immunisations.

Mice

Female or male C3H/HeN (H-2^k) and C3H/HeJ [LPS hyporesponsive (H-2^k)] mice were obtained from Harlan UK Limited (Blackthorn,UK). The animals were kept under specific pathogen free conditions and the mice were used between 8-12 weeks.

Immunisations

C3H/HeN and C3H/HeJ mice were immunised by the intra peritoneal route with 5µg of *K. pneumoniae* capsular polysaccharides in 500µl PBS. Control mice received PBS only. The immunisation schedule is shown in Figure 2.1.

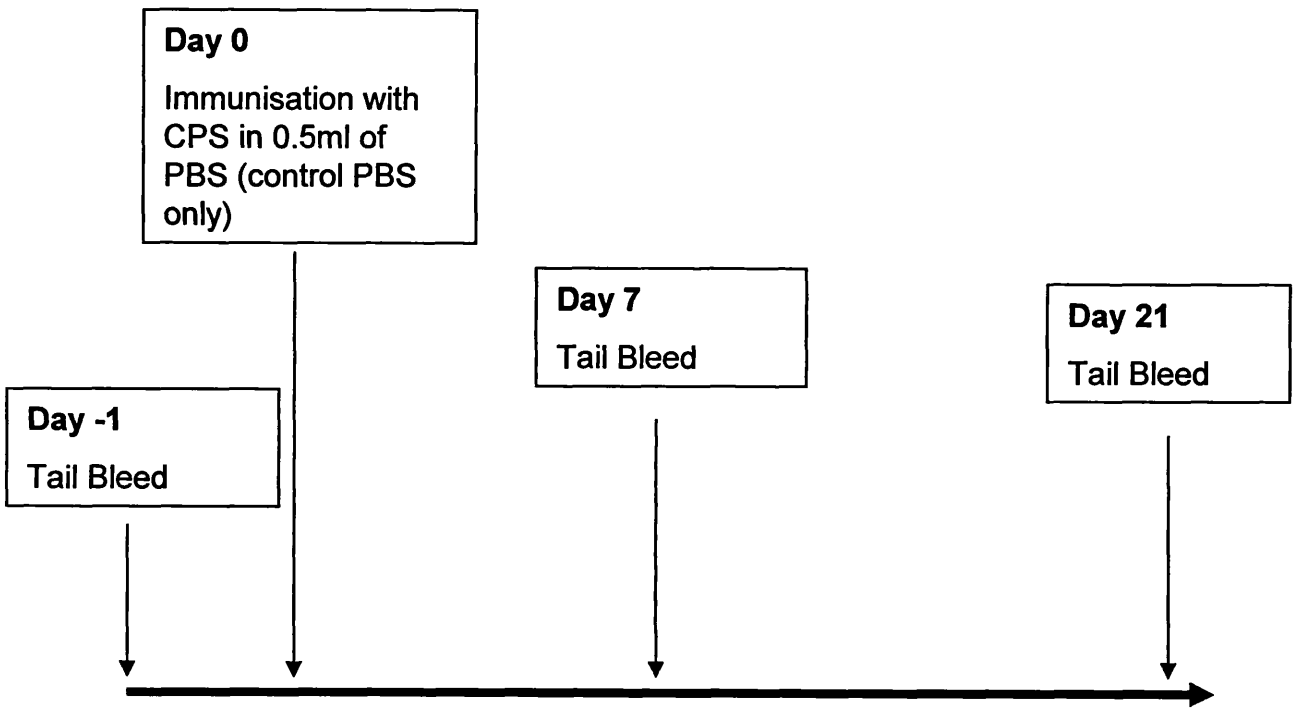


Figure 2.1 Schedule for immunisation of mice with *Streptococcus pneumoniae* and *Klebsiella pneumoniae* capsular polysaccharides and serum collection.

2.2.4c Protocol of chicken gamma globulin (CGG) immunisations

Mice

Female or male C3H/HeN and C3H/HeN mice aged 8-12 weeks were obtained from Harlan UK limited (Blackthorn, UK). Animals were kept under specific pathogen free conditions.

Removal of lipopolysaccharide from CGG

Before immunisation, contaminating LPS was removed from the CGG preparation using a Vivapure Mini (Q) spin column [quaternary ammonium strong basic anion exchanger (Vivascience Ltd, UK)]. CGG [500ul (5mg/ml in PBS)] was loaded onto the column and then centrifuged at 2000 x g for 10 mins in a Beckman microfuge (model:microfuge 18). The negatively charged LPS bound to the column whereas the positively charged CGG was eluted in PBS. The eluted sample was collected and LPS removal was confirmed by the LAL assay as described in Section 2.2.3e.

Immunisations

Mice were immunised by the intra peritoneal route with 100µg of CGG in 500µl PBS with or without adjuvant. When injected with Titermax, an equal volume of CGG in PBS was emulsified with the adjuvant before injection. Titermax contains three ingredients: a block polymer, CRL-8941, squalene and a metabolizable oil. The identities of the polymer and the oil in Titermax have not been disclosed. The potency of Titermax lies in its immunostimulatory activity of its components and in the fact that it forms a

stable water-in-oil emulsion. For other adjuvants tested, such as the LPS and CPS from *K. pneumoniae*, a set amount of adjuvant (5-25µg) was mixed with 100µg of CGG prior to injection. The immunisation schedule is shown in Figure 2.2.

2.2.4d Preparation of mouse serum

Mice were bled from the tail and blood was collected. Blood was left for 1h at RT and then left overnight at 4°C. In order to clarify serum from coagulated blood, samples were centrifuged at 2500 x g for 10 mins in a Beckman microfuge (model:microfuge 18). Serum was removed and placed into new tubes and then stored at -18°C until required.

2.2.4e Characterisation of serum antibody to *Streptococcus pneumoniae* capsular polysaccharides by ELISA.

The purified pneumococcal CPSs may contain a certain amount of pneumococcal C polysaccharide or cell wall polysaccharide (CW-PS) which is a constituent of the pneumococcal cell wall. It is recommended to absorb sera with C polysaccharide to remove antibodies to this polysaccharide when testing for antibodies to pneumococcal CPS antigens (Aaberge et al., 1993). Mouse antisera were absorbed with 100µg/ml of CW-PS (Statens Serum Institute, Copenhagen, Denmark). Absorption was carried out for 1h at RT at a 1 in 50 serum dilution in PBS + 0.05% Tween 20, prior to further dilution in buffer without CW-PS.

Immunoplates were coated with 50µl/well of CPS 10µg/ml in 0.9% (w/v) NaCl and sealed with parafilm prior to incubation in a damp box overnight at 37°C. The plates were then washed 3 times with wash buffer [PBS + 0.05% Tween 20]. Coated plates were blocked for 1h at RT with 100µl of PBS + 4% milk

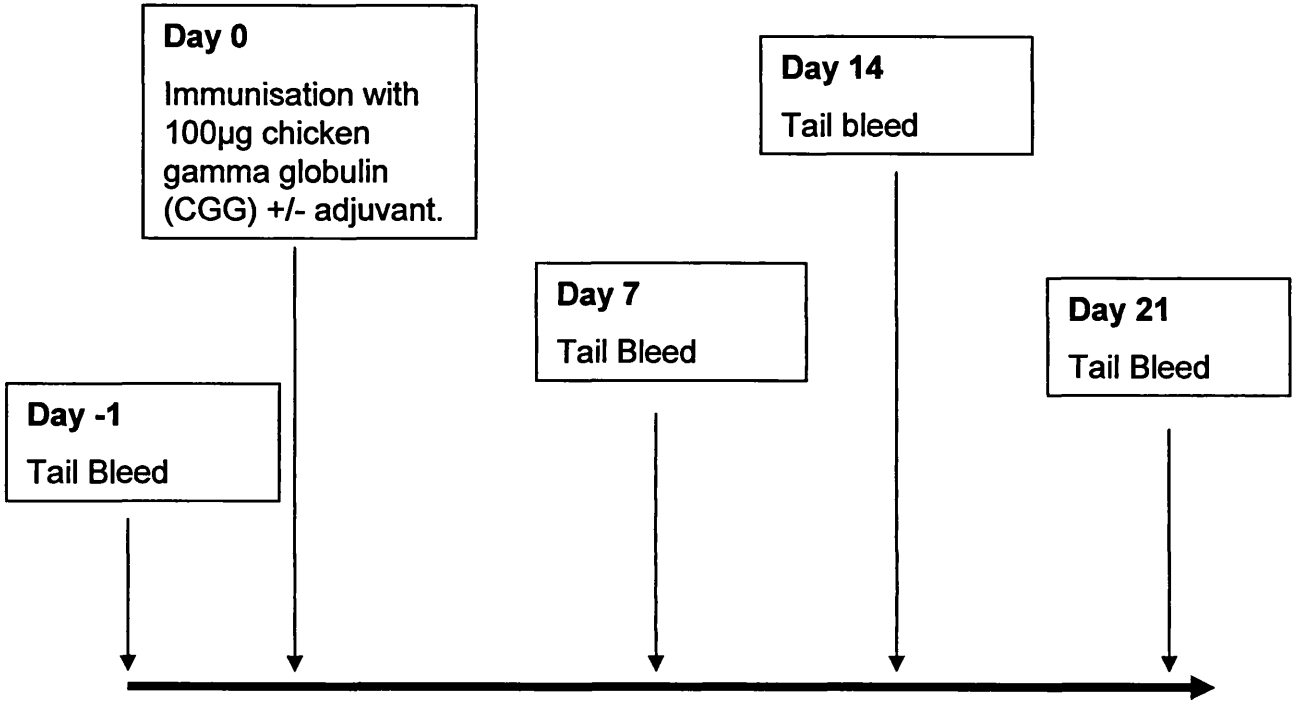


Figure 2.2 Schedule of immunisations of mice with chicken gamma globulin (CGG) and serum collection.

powder. Plates were then washed 3 times. The CW-PS absorbed mouse sera (as described previously) were serially diluted starting at 1 in 50. Diluted antiserum (50µl) was added to duplicate wells and incubated at RT for 2h in a moist box. Plates were washed again, followed by incubation with 50µl/well of the secondary antibody (see Section 2.1.1b) diluted 1 in 500 in PBS + 0.05% Tween 20 for 2h at RT. The plates were washed then 50ul/well of substrate solution o-phenylenediamine dihydrochloride [OPD (horseradish peroxidase substrate)] or p-nitro-phenyl-phosphate [pNpp (alkaline phosphatase substrate)] was added. Reactions were stopped with 15µl/well of either 3M H_2S0_4 (OPD) or 3M NaOH (pNpp). Optical densities were read at 490nm (OPD) or 405nm (pNpp) on a plate reader [spectraMAX 340 (Molecular Devices)]

2.2.4f Characterisation of serum antibody to *Klebsiella pneumoniae* capsular polysaccharides by ELISA.

Immunoplates were coated with 10µg/ml of CPS (extracted and purified as described in Section 2.2.1) in 0.9% (w/v) NaCl in a damp box overnight at 37°C. Plates were washed with wash buffer, and then blocked with 100µl/well of PBS + 4% milk powder for 1h at RT. Plates were washed and then mouse sera were serially diluted, starting at a 1in 50 dilution and then added to wells (50µl/well) and incubated at RT for 2h. After washing, 50µl of secondary antibody was added to each well (see Section 2.1.1b) and incubated for 2 hours at RT. The secondary antibody was diluted 1 in 500 in PBS + 0.05% Tween 20. Plates were washed and then 50µl/well of OPD peroxidase substrate was added. After 5 minutes the reaction was stopped with 15µl/well of 3M H_2S0_4 and plates were read at 490nm on a plate reader.

2.2.4g Characterisation of serum antibody response to chicken gamma globulin (CGG) by ELISA.

Immunoplates were coated with 50µl/well CGG [5µg/ml in 0.1M sodium carbonate buffer (pH9.6)] obtained from Jackson Immunoresearch, Cambridge, UK. Plates were placed in a moist box and incubated overnight at RT. Plates were then washed 3 times with wash buffer [PBS + 0.05% Tween 20] and then blocked with 100µl/well of PBS + 4% milk powder for 1 hour at RT. After washing, mouse antisera were diluted with PBS + 0.1% Tween 20 in serial 2-fold dilutions. Diluted antisera (50µl) was added to wells in duplicate and incubated at RT for 2h in a moist box. The plates were then washed with wash buffer. The secondary antibody (50µl/well) (see Section 2.1.1b in Materials and Methods) was diluted in PBS + 0.05% Tween 20 was added to wells, and incubated at RT for 2h. After washing, 50µl/well of OPD peroxidase substrate was added. The reaction was stopped after 5 mins with 15µl/well of 3M H_2S0_4. Plates were read at 490nm on a plate reader [SpectraMAX 340 (Molecular Devices)]

2.2.5 *In vitro* cell assays

2.2.5a Cell proliferation assay using ^{3}H thymidine

Spleens from naïve mice were removed aseptically. A single cell suspension was produced by gently pushing the spleen through a cell strainer (40µm) using a barrel of a 5ml syringe. The cell suspension was then centrifuged (Sorvall RT7 plus) at 100 x g for 5 mins at 4°C. The cells were washed twice with complete RPMI [c.RPMI (RPMI 1640 with 1% penicillin/streptomycin and 10% FCS)], and trypan blue was used to confirm viability. Cells were re-

suspended in c.RPMI to a density of 2 x 10^6 cells/ml. The cell suspension (100µl) was dispensed to each well of a 96 well U-bottomed plate. For stimulation 100µl of test sample in c.RPMI was added in triplicate. Phorbol myristate acetate (PMA) at 5ng/ml and Ionomycin (200ng/ml) were used as a positive control, whereas medium only was used as a negative control for cell proliferation. The cells were then incubated at 37°C, 5% CO_2 for 72h. Tritiated thymidine (Amersham Pharmacia Biotech) at 1µCi in 20µl c.RPMI was then added to each well, followed by a 12h incubation as above. Plates were harvested using a TOMTEC 96-well harvester. The filters were dried and sealed in a plastic bag with Betaplate scintillant. Incorporated [^{3}H] thymidine was measured using a MicroBeta TRILUX counter (Wallac, Turku, Finland).

2.2.5b Cytokine release assay

Mouse splenocytes were prepared and stimulated following the same method as described in Section 2.2.5a for thymidine proliferation assays. Samples were tested in duplicate and supernatant was harvested after 18h, 24h, 48h and 72h. Supernatants were stored at -18°C until used for cytokine analysis.

2.2.5c CFSE labelling of mouse splenocytes

Cells to be labelled were resuspended at 1 x 10^7 cells/ml in PBS + 0.01% BSA. A 5mM stock solution of carboxyfluorescein diacetate succinimidyl ester (CFSE) in DMSO was added to a final concentration of 5µM and incubated at 37°C for 10 mins. At the end of the incubation period, the cells

were immediately washed with cold PBS + 5% FCS and passed through a 40µm filter. Cells were washed 3 times with PBS and then counted.

2.2.5d Cytokine detection

Cytokine Cytometric Bead Array (CBA) assay

Cytokines present in the supernatants from stimulated mouse splenocytes were detected quantitatively using a cytokine cytometric bead array (BD Biosciences Pharmingen). The mouse TH1/TH2 CBA was used to detect TNF-α, IFN-γ, IL-5, IL-4 and IL-2 and the mouse inflammation CBA to detect TNF-α, IFN-γ, IL-10, IL-6, IL-12p70 and MCP-1. The assay was carried out according to manufacturer's instructions. Samples were examined by flow cytometry using a FACScalibur flow cytometer. Data was analysed using BD CBA software (BD Biosciences Pharmingen).

2.2.5e Immunofluorescence staining of cell surface markers

Prior to labelling, cells were incubated at 10^7cells/ml in blocking buffer (PBS + 1% BSA + 0.01% sodium azide) for 30 mins at 4°C to minimise non-specific binding. Antibodies directed to cell surface markers (see Section 2.1.1a) were diluted in the blocking buffer. Cells (10^6) were labelled with 50µl of diluted antibodies for 15 mins at 4°C in the dark, followed by two washes with blocking buffer. Labelled cells were examined by flow cytometry using a FACScalibur flow cytometer in conjunction with the CellQuest software package (Becton Dickinson, Oxford, UK).

2.2.6 Statistics

Data were analysed using software packages Microsoft Excel 2002 and Minitab 13 for Windows. Statistical analysis was performed according to directions given by Dr. Sandro Leidi of the University of Reading Statistical services centre. Statistical significance between two independent groups was performed using Two Way ANOVA (Analysis of Variance) whereas multiple groups were analysed using a General Linear Model ANOVA.

Chapter Three

Results

Chapter Three

Purification and characterisation of capsular polysaccharides from *Klebsiella pneumoniae*

3.1 Introduction

In order to address the potential biological effects of *K. pneumoniae* CPS it is essential to have pure preparations because contaminating components may have an influence on the results generated from immunological studies. It is particularly problematic when the component needs to be purified from complex mixtures as found in bacterial extracts. Common contaminants of CPS preparations include LPS, DNA, and lipoproteins, and these "pathogen-associated molecular patterns" can have a major effect upon the immune system. Toll-like receptors function as pathogen recognition receptors in mammals and play an essential role in the recognition of these and other microbial components (Akira, 2001). Recognition often results in a host inflammatory response. It is therefore critical that these potential contaminants are removed from CPS preparations to be investigated.

LPS is a major component of the outer membrane of Gram negative bacteria. LPS exhibits a variety of biological activities, such as toxicity, adjuvanticity, complement activation, polyclonal B cell activation, and cytokine-inducing activities (Morrison and Ulevitch, 1978; Morrison and Ryan, 1979). The removal of LPS from test samples prior to immunological assays in thus very important. For example it was shown that endotoxin contamination in recombinant human heat shock protein 70 preparation was

responsible for the induction of TNFα release from murine macrophages (Gao and Tsan, 2003). This example highlights the effect LPS can have on experimental results. The potential presence of LPS has also complicated the interpretation of previous results on the biological effects of CPS from *K. pneumoniae*. The CPS and LPS share common localisation at the cell wall surface. Moreover, the spontaneous release of LPS by bacteria is well established (Cadieux et al., 1983) and contributes to contamination during CPS extraction. For this study a number of methods have been tried for CPS purification. Chemical treatments such as alkaline de-O-acylation, which selectively removes O-acyl fatty acids, can be effective in detoxifying LPS (Seid and Sadoff, 1981). However this method can cause degradation of the polysaccharide, depending on the polysaccharide structure. Ultracentrifugation, a method commonly used to recover LPS, does not significantly remove LPS from *K. pneumoniae* CPS (Cryz et al., 1985). A method that we have found more successful is gel filtration under dissociating conditions (elution buffers containing detergents). The original method as described by Adam et al. (1995) required elution at high pH and high temperature which we found could degrade CPS samples. Therefore the method was modified to use buffers at pH 7 which was then used for subsequent routine purifications (Zamze et al., 2002).

3.2 Objectives

The objective of the work described in this chapter was to produce a CPS preparation that was free from as many other known biologically active components as possible by improving methods of *K. pneumoniae* CPS purification.

3.3 Purification of *Klebsiella pneumoniae* capsular polysaccharides

3.3.1 *Klebsiella pneumoniae* CPS extraction

CPSs from a number of different serotypes of *K. pneumoniae* were obtained using the extraction and purification methods described in Section 2.2.1. The CPS extracts were subjected to nuclease, protease and ultracentrifugation treatment and then quantified for CPS, protein, nucleic acid and LPS content. Table 3.1 shows the typical content by weight of a CPS extract.

Table 3.1 Typical contents of a CPS extract
CPSs were extracted and then purified by ultracentrifugation, and digested with both protease and nuclease. Preparations were then analysed for LPS, protein and nucleic acid content.

Component	CPS extract (typical % content)
Protein	10-15%
Nucleic acid	5%
LPS	1-2%

3.3.2 Size fractionation and purification

CPS extracts were then fractionated by gel filtration chromatography (TSK G5000). Figure 3.1 shows two examples of typical gel filtration profiles of *K. pneumoniae* CPS extracts. The carbohydrate eluted at two time points, as shown by refractive index (Figure 3.1, peaks 1 and 2). The first peak, labelled 1, was a high molecular weight (MW) component with elution volume slightly less than the void volume of the column. CPS quantification by phenol

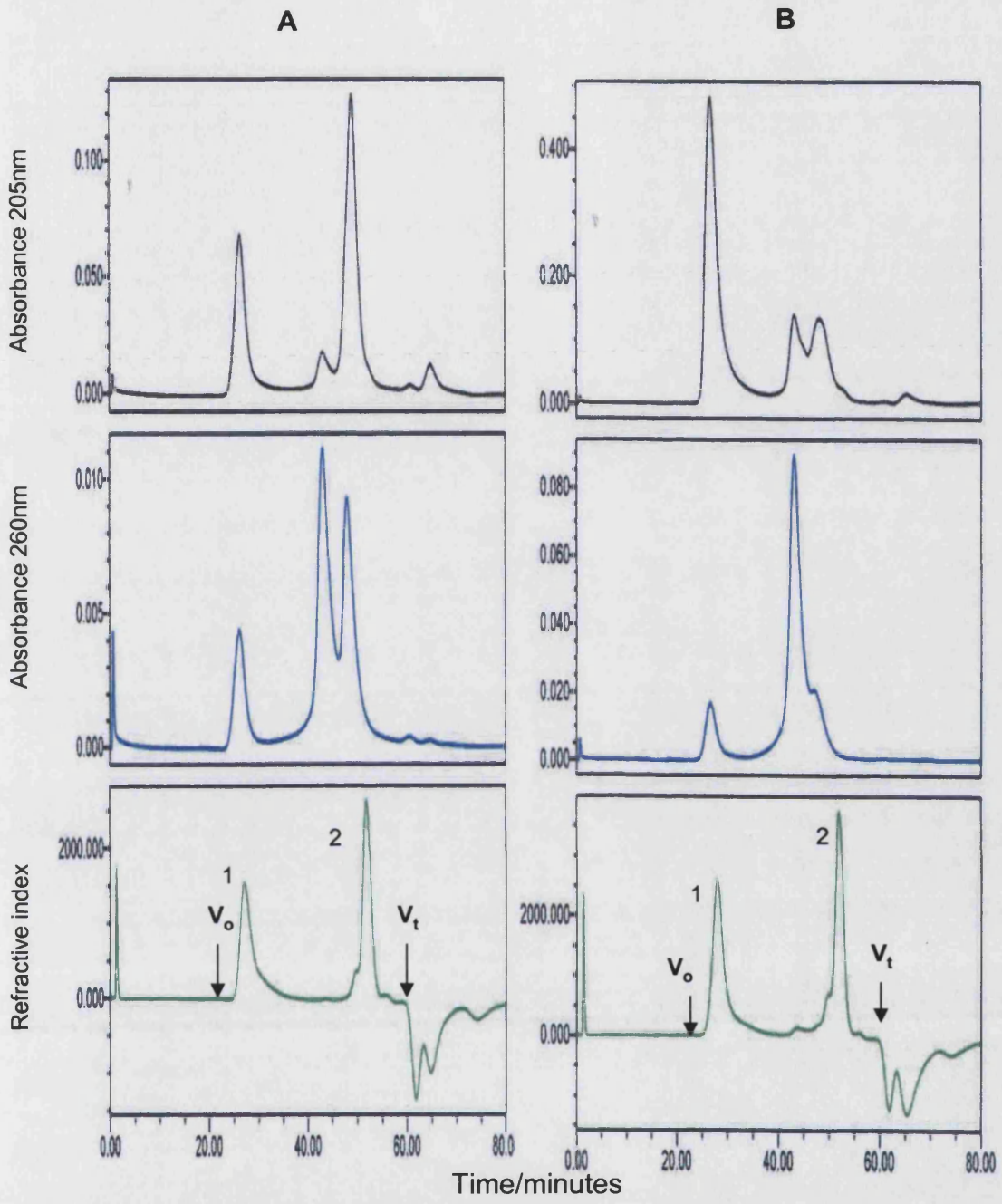


Figure 3.1 Gel filtration profiles of *Klebsiella pneumoniae* CPS serotypes K26 and K27.

K. pneumoniae CPSs were extracted and purified as described in Section 2.2.1. Chromatography was performed on a TSK G5000 column in PBS. Void and total volume (V_o and V_t respectively) of this column are indicated. The flow rate was 0.25ml/minute. The refractive index and the absorbance of the eluant at 205nm and 260nm were monitored for *K. pneumoniae* CPSs (A) K26 and (B) K27. Peaks 1 and 2 were pooled separately. Pool 1 was shown to contain 90% of the total carbohydrate loaded onto the column by the phenol sulphuric acid assay (Section 2.2.3b).

sulphuric acid assay showed that this peak contained almost 90% of the total carbohydrate recovered from the column. The second lower MW peak contained only a small amount of carbohydrate. All CPS extracts gave very similar results. The CPS extracts also contained protein and/or nucleic acid contamination, as shown by the 260nm absorbance profile. The MW of the CPS was also determined using the gel filtration column. Dextrans of known molecular weights (40,000, 70,000, 200,000, 500,000, 1,800,000) were used to calibrate the column as shown in Figure 3.2. The 1,800,000 MW dextran standard eluted at approximately 30 mins, similar to the elution time of CPS peak 1 (Figure 3.1). CPSs eluting in peak 1, were therefore estimated to have MW of approximately 2×10^6 Da.

Gel filtration profiles of CPS extracts following ultracentrifugation, nuclease and protease digestion are shown in Figure 3.3. The 260nm absorbance trace, which detects the presence of DNA and protein, varied for each of the samples. When the CPS extract was not subjected to ultracentrifugation or protease and nuclease digestion there were much more high MW protein and nucleic acid present as shown by the peaks at 20-30 mins. The samples subjected to ultracentrifugation, protease and nuclease digestion contained lower MW protein and nucleic acid, which eluted later than the carbohydrate (i.e. at 40-60mins). Separating CPS extracts by gel filtration chromatography allows the carbohydrate in the first peak to be pooled and most of the protein and nucleic acid to be removed from the sample.

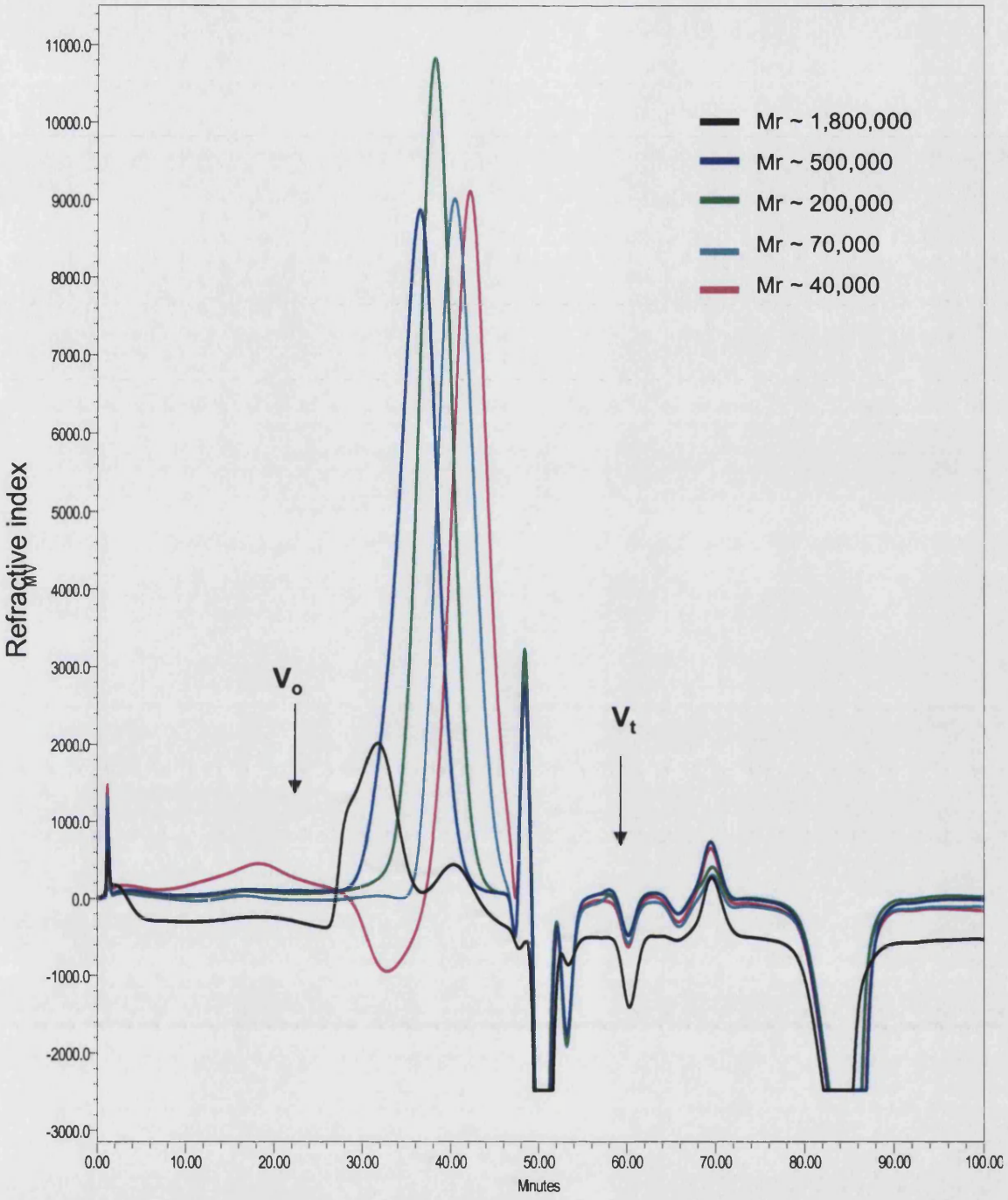


Figure 3.2 Gel Filtration profile of dextran standards.

Known molecular weights of dextran (40,000, 70,000, 200,000, 500,000,1,800,000) from Leoconostoc ssp. were analysed by gel filtration chromatography on a TSK G5000 column in PBS containing 0.25% (w/v) sodium deoxycholate at 60°C. Void and total volume (V_o and V_t respectively) of the column are indicated. The refractive index of the eluant was monitored.

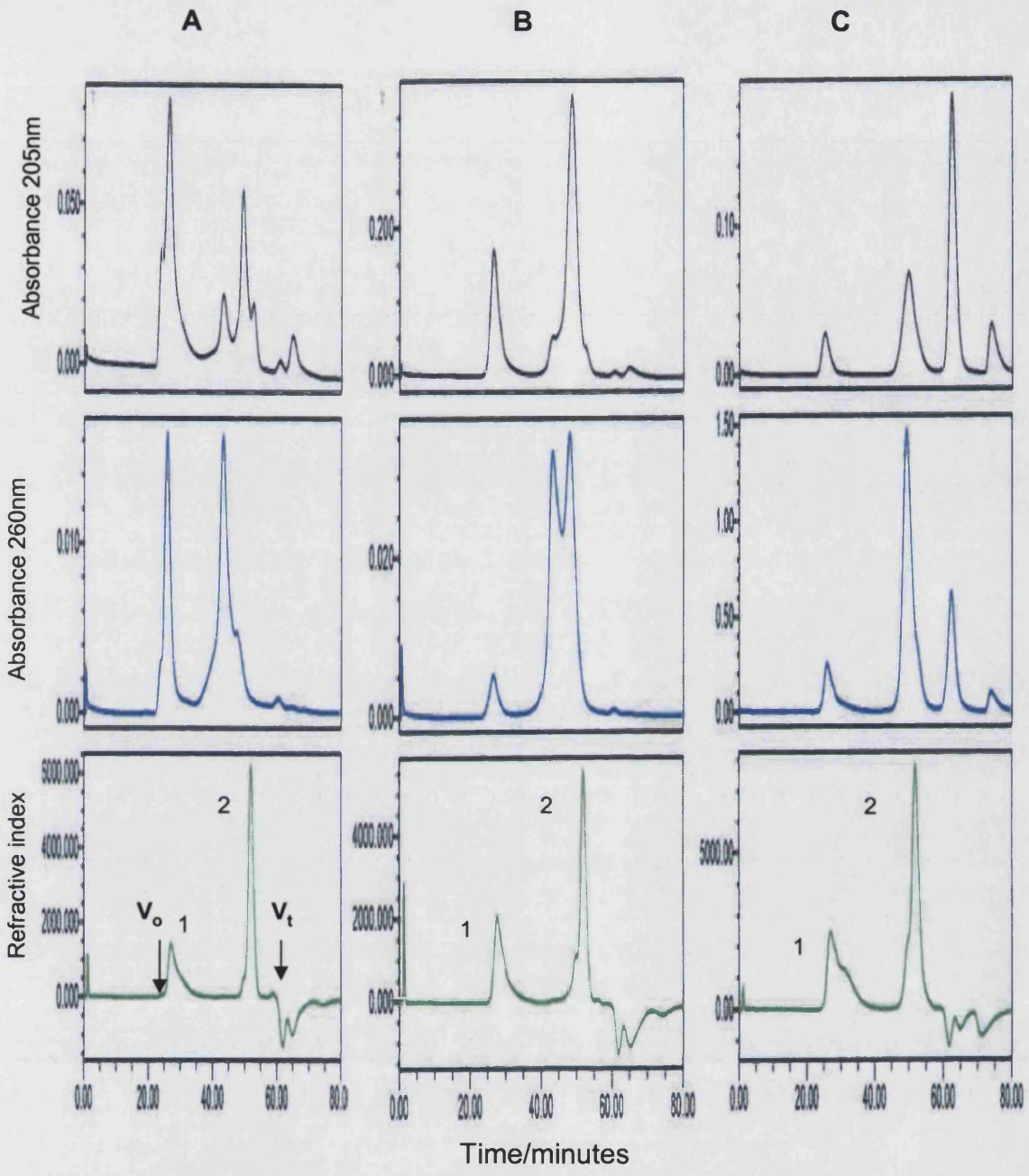


Figure 3.3 Gel filtration profiles of *Klebsiella pneumoniae* CPS serotype K11 subjected to different degrees of purification.

K. pneumoniae CPS K11 was subjected to different degrees of purification (A) no protease, nuclease or ultracentrifugation, (B) protease treatment, no nuclease or ultracentrifugation, (C) protease, nuclease and ultracentrifugation treatment. Chromatography was carried out on a TSK G5000 column in PBS. Void and total volume (V_o and V_t respectively) of the column are indicated. The flow rate was 0.25ml/min. The refractive index and the eluant absorbance at 205nm and 260nm were monitored. Peaks 1 and 2 were pooled separately and analysed for carbohydrate content as described in Section 2.2.3b. Pool contained 90% of the total carbohydrate loaded onto the column.

3.3.3 Removal of LPS from CPS extracts.

The following methods were attempted or used to remove LPS from the CPS extracts.

3.3.3a Triton X-114

Extraction of LPS by Triton X-114 was carried out as described in Section 2.2.1f. There was a ten fold decrease in the amount of LPS present in the CPS extract as shown by the LAL assay, but there was a 50% loss of carbohydrate. Due to the loss of large amounts of CPS, this method was not used further.

3.3.3b Gel filtration chromatography under dissociating conditions.

When PBS was used as an elution buffer, LPS formed high MW micelles and co-eluted in pool 1 (high MW peak) with CPS. It was possible to disrupt micelle formation and therefore separate CPS and LPS using dissociating conditions. K55 LPS (Figure 3.4) was separated by gel filtration chromatography in PBS and also by published conditions using a dissociating buffer [10mM Tris-HCl (pH8.7) containing 0.2M NaCl, 1 mM EDTA and 0.25% (w/v) sodium deoxycholate] at 60°C (Adam et al., 1995). This allowed comparison of the refractive index traces, for each elution buffer, in order to determine at which time point LPS eluted from the column. When PBS was used as an elution buffer, LPS eluted at around 30 mins (indicated by A in Figure 3.4). When a dissociating buffer was used LPS eluted much later from the column at around 40-50 mins. This is indicated on the refractive index profile as B. In non-dissociating conditions CPS and LPS co-eluted, whereas under dissociating conditions LPS eluted much later.

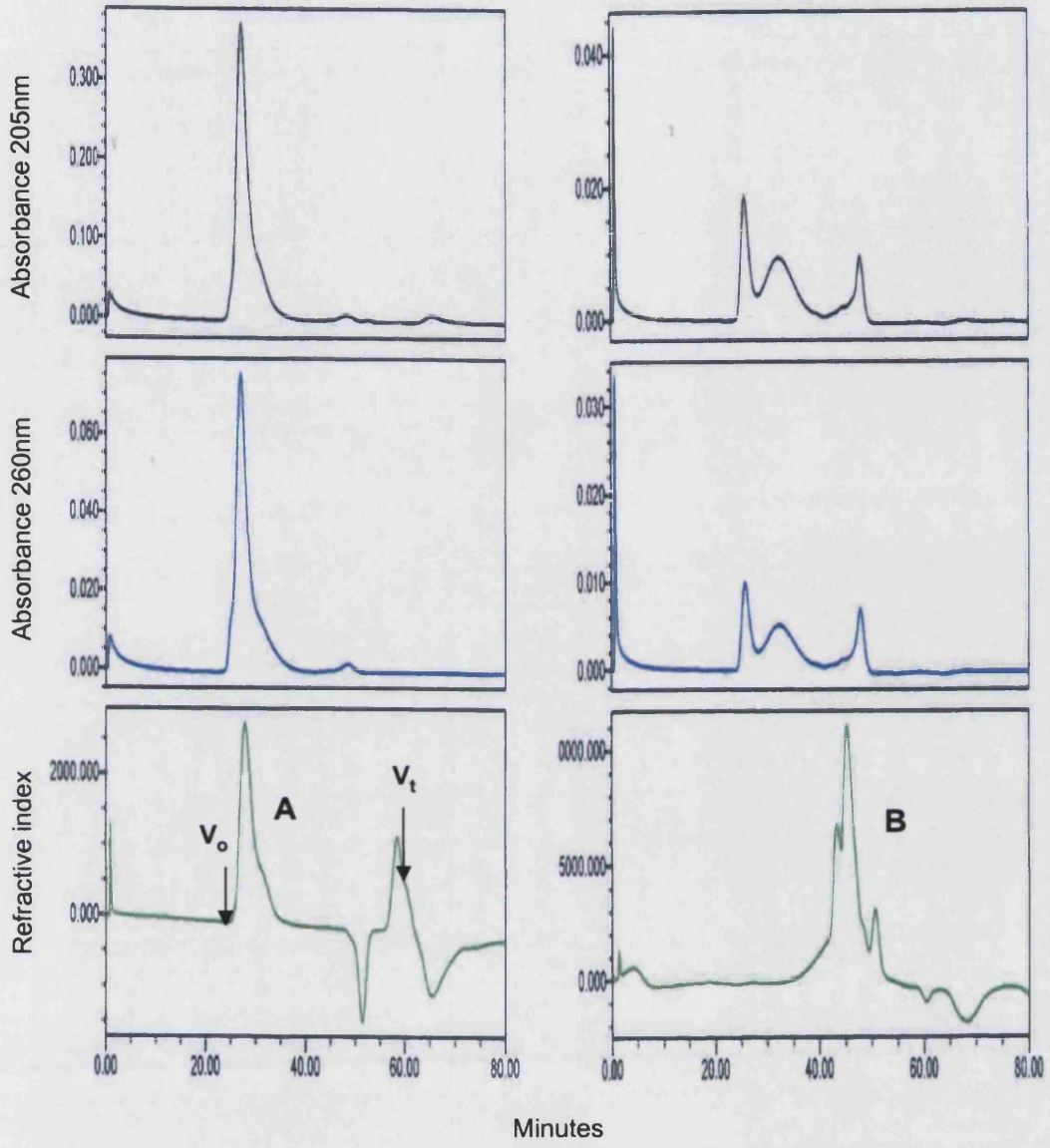


Figure 3.4 Gel filtration profiles of *Klebsiella pneumoniae* serotype K55 LPS (TSK G5000 column)

K. pneumoniae LPS was obtained by aqueous 45% (w/v) phenol extraction as described in Section 2.2.2a. LPS was then analysed by gel filtration chromatography on a TSK G5000 column in either PBS (A) or a dissociating buffer [10mM Tris-HCl (pH8.7) containing 0.2M NaCl, 1mM EDTA and 0.25% (w/v) sodium deoxycholate] at 60°C (B). Void and total volume (V_o and V_t respectively) of the column are indicated. The refractive index and the absorbance of the eluant at 205nm and 260nm were monitored.

Since LPS and CPS eluted at different times from the column, so this was a good method for removing LPS from CPS extracts.

3.3.4 Gel filtration chromatography of *Klebsiella pneumoniae* CPS extracts and further analysis of pooled samples.

Using relatively high pH and temperature to elute CPS can lead to the loss of alkali labile substituents such as O-acetyl groups (many CPS contain these groups, e.g *K. pneumoniae* serotype K55) and partial de-O-acylation of lipids, which may be important if the CPS has a lipid-anchor. This could therefore modify the structure of the CPS and likely change its antigenicity or immunogenicity. Different conditions of temperature and pH were therefore investigated. CPS extracts were separated by gel filtration chromatography (TSK G5000) using the elution buffers shown in Table 3.2. Fractions were collected every 2 mins. The two carbohydrate peaks were pooled and subjected to further analysis.

3.3.4a Recovery and MW of polysaccharide

The refractive index profiles generated after gel filtration of CPS with different dissociating buffers were identical. Peak one eluted at 30 mins which confirmed that there was no gross change in the MW of the polysaccharide when dissociating buffers were used. To recover the samples, pools 1 and 2 were dialysed extensively against water, at RT and freeze dried.

Table 3.2 Gel filtration elution buffers

Buffer	CONDITIONS: Components / temperature / pH
A	PBS 30°C

B*	(Sodium deoxycholate buffer)10mM Tris-HCl **pH8.7** containing 0.2M NaCl, 1mM EDTA and 0.25% (w/v) sodium deoxycholate at **60°C**
C*	(Sodium deoxycholate buffer)10mM Tris-HCl **pH8.7** containing 0.2M NaCl, 1mM EDTA and 0.25% (w/v) sodium deoxycholate at **30°C**
D*	(Sodium deoxycholate buffer) 10mM Tris-HCl **pH7.5** containing 0.2M NaCl, 1mM EDTA and 0.25% (w/v) sodium deoxycholate at **30°C**
E*	(Sodium deoxycholate buffer) 10mM PBS **pH 7.0** containing 0.25% (w/v) sodium deoxycholate at **60°C.**

Residual detergent was removed by precipitation with 80% (v/v) ethanol. Total carbohydrate in pools 1 and 2 was quantified by the phenol sulphuric acid assay. The recovery of carbohydrate under all conditions was 80% or greater. For CPS extracts that were eluted with PBS, the recovery was slightly greater than that seen for dissociating buffers, probably due to losses at the precipitation step.

3.3.4b Effect of separating conditions on CPS antigenicity

The effect of using different elution buffers for gel filtration on CPS antigenicity was investigated by ELISA using anti-CPS specific rabbit antisera. ELISA assays were carried out as described in Section 2.2.3a. Plates were coated with control CPS and CPS pool 1 recovered from each of the different elution conditions. These were then incubated with rabbit anti-

capsular serum specific for K55 serotype. Figure 3.5 shows the change in anti-capsular antibody binding to K55 CPS extracts eluted with different buffers.

The absorbance at 490nm was plotted against reciprocal serum dilution for each elution condition. Absorbance values indicate the amount of binding of antibody to antigen. If the antigen was modified a reduction in antibody binding was expected and thus a loss in antigenicity. When K55 CPS extracts were eluted from the column with PBS containing 0.25% (w/v) sodium deoxycholate there was no change in absorbance levels when compared to the control CPS. This suggests no or very little modification of the CPS structure. For other buffers used there was some reduction in absorbance levels compared to control CPS, especially the Tris/ deoxycholate buffer at pH 8.7 and 60°C. The high temperature and pH may have modified the CPS and affected the binding of the specific anti-capsular antiserum. It was therefore decided that PBS containing 0.25% (w/v) sodium deoxycholate would be used to separate CPS extracts by gel filtration chromatography because no effect on the antigenicity of the CPS was detected.

3.3.4c Analysis of CPS purified by dissociating conditions

A representative profile of CPS fractionated using PBS, 0.25% sodium deoxycholate at 60°C is shown in Figure 3.6. The carbohydrate, as with using PBS alone, eluted in two peaks and these two peaks were pooled. Pool 1 which contained 90% of carbohydrate recovered from the column was further analysed for protein, LPS and nucleic acid content. Table 3.3 shows the

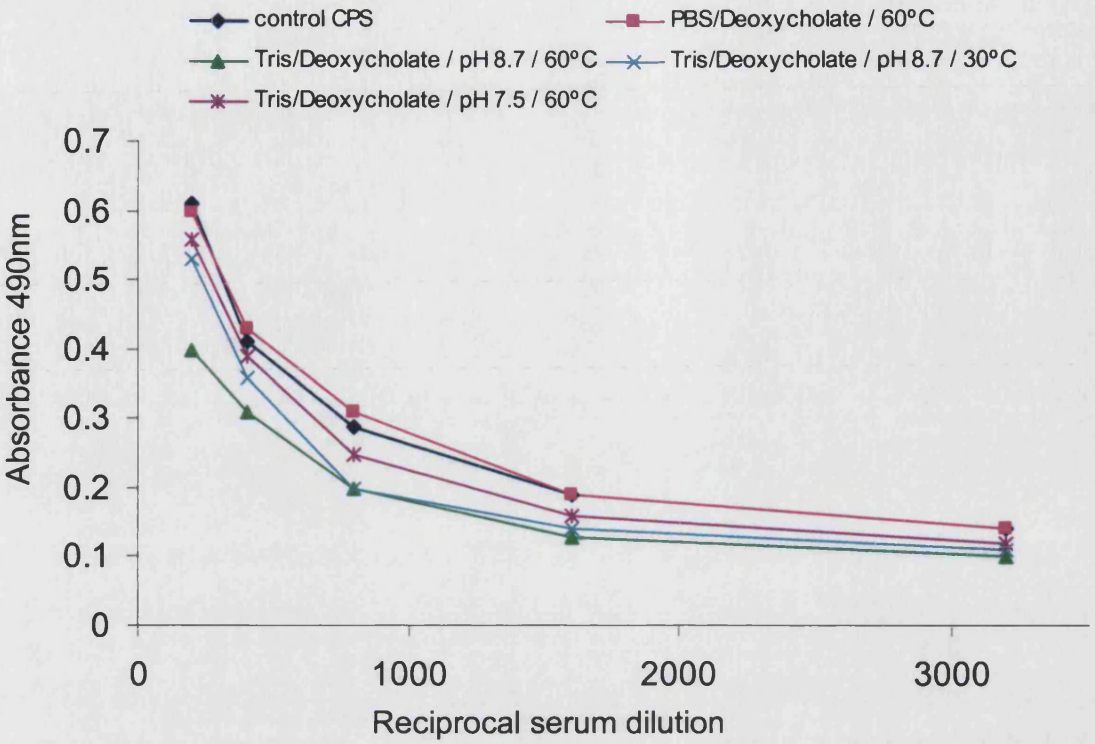


Figure 3.5 The binding of rabbit anti-capsular antiserum to *Klebsiella pneumoniae* serotype K55 (pool 1) following gel filtration.

K. pneumoniae CPS K55 was extracted and purified as described in the methods section. Chromatography was carried out on a TSK G5000 column with different elution buffers (Table 3.2). Peak one was pooled for each elution condition, dialysed against water, and freeze-dried, and detergent was removed by precipitation with ethanol. The binding of rabbit anti-capsular antiserum to K55 was determined by ELISA using K55 CPS extract not subjected to gel filtration as a control CPS. Anti-rabbit IgG (γ-chain specific) HRP conjugate was used to detect capsular antiserum and OPD was used as a substrate. Absorbance was read at 490nm. These data are representative of other CPS extracts.

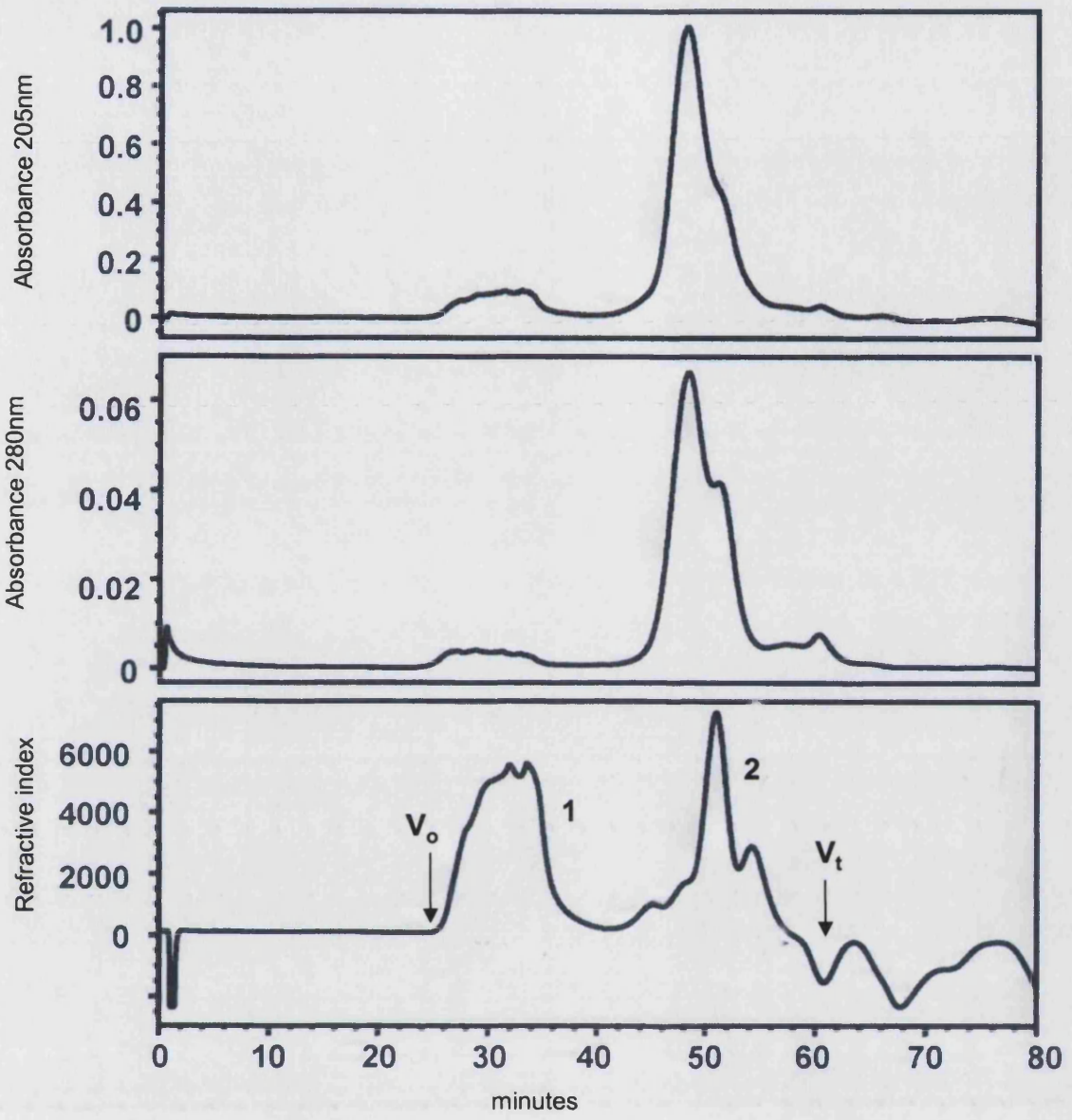


Figure 3.6 Gel filtration profile of *Klebsiella pneumoniae* CPS serotype K31.

K. pneumoniae CPS was extracted and purified as described in the Section 2.2.1. Chromatography was carried out on a TSK G5000 column in PBS containing 0.25% (w/v) sodium deoxycholate at 60°C. The flow rate was 0.25ml/minute. The refractive index and the absorbance of the eluant at 205nm and 280nm were monitored. Peaks 1 and 2 were pooled and detergent was removed. Pool 1 was shown to contain 90% of the total carbohydrate recovered fro the column by the phenol sulphuric acid assay (Section 2.2.3b).

typical content by weight of pool 1 from a CPS extract separated by gel filtration chromatography under dissociating conditions. There was a large reduction in protein, nucleic acid and LPS content in CPS pool 1 compared to the CPS extract (Table 3.1 and 3.3). CPS pool 2 was analysed for protein and LPS. The LAL assay confirmed that almost all of the LPS were found in pool 2.

Table 3.3 Typical contents of CPS pool 1
CPS extract was separated on a TSK G5000 column in PBS containing 0.25% (w/v) sodium deoxycholate. The two carbohydrate peaks were pooled and then subjected to LPS, protein and nucleic acid content analysis. Pool 2 contained most of the protein and LPS from crude preparations.

Component	Pool 1 typical % content
Protein	1%
Nucleic acid	< 0.1% or non detectable
LPS	0.01%

3.4 Characterisation of *Klebsiella pneumoniae* capsular serotypes by SDS-PAGE

CPS extracts were analysed by SDS-PAGE using staining methods for detection of CPS, LPS and protein. *K. pneumoniae* CPS extracts were analysed by SDS-PAGE using 10% separating gels as described in Section 2.2.3g. In some cases extracts were also analysed by western blotting using rabbit anti-CPS antisera. Figure 3.7 shows the SDS-PAGE and western blot profiles obtained for serotypes K31 and K3 crude extracts. Most of the capsular serotypes studied gave a variety of banding patterns, although all

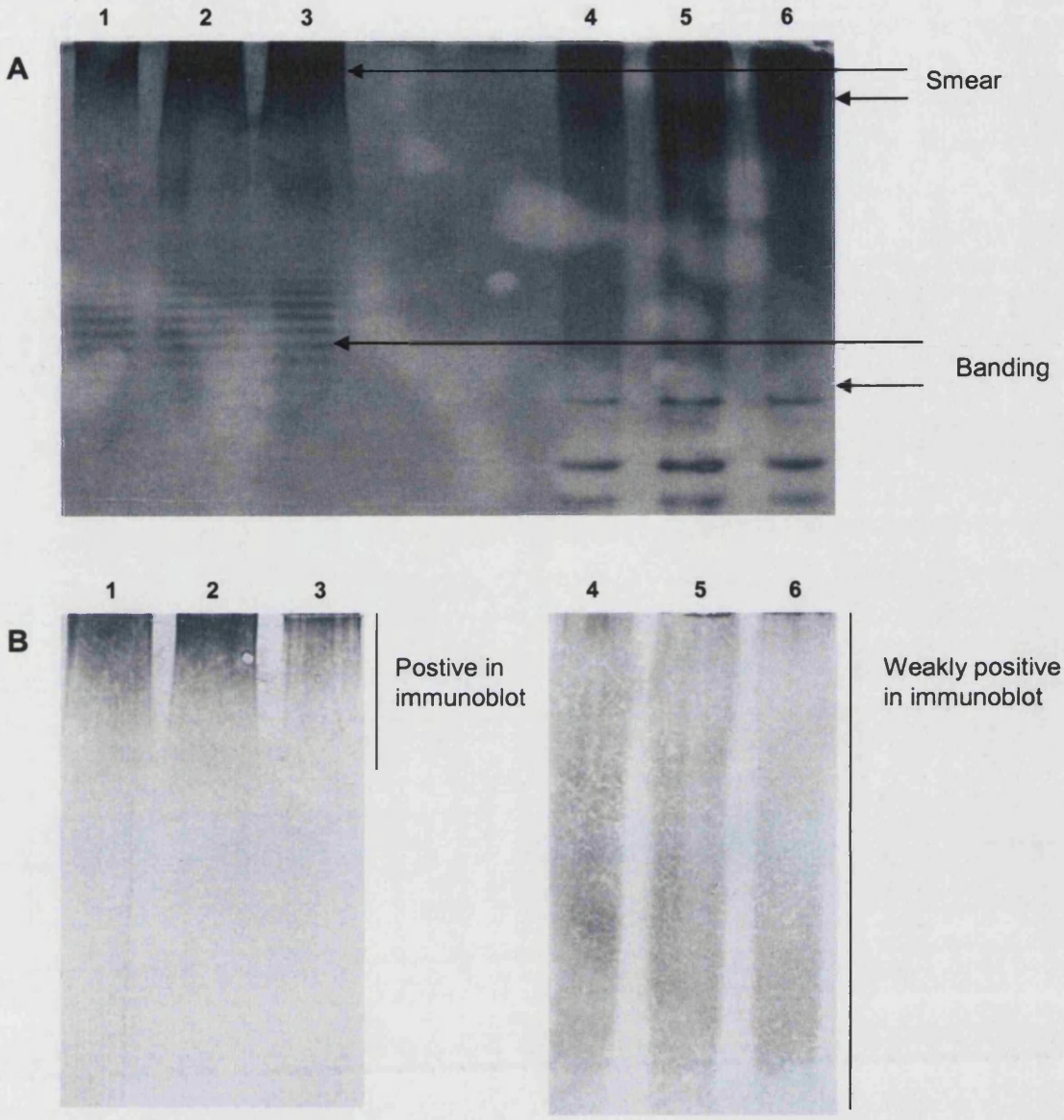


Figure 3.7 SDS-PAGE and western blot analysis of *Klebsiella pneumoniae* CPS serotypes K31 and K3.

K. pneumoniae CPSs were extracted as described in Section 2.2.1b. CPS extracts (before gel filtration) were then analysed by SDS-page (A), followed by western blotting (B). Lanes 1-3 contain K31 CPS extract (5μg) and lanes 4-6 contain K3 CPS extract (5μg). Separation was on a 10% (w/v) gel and detection with Alcian blue followed by silver staining. For western blotting, CPS was transferred from a 10% gel onto a Nytran membrane and then detected with specific rabbit anti-capsular antisera followed by anti rabbit IgG HRP conjugate. Blots were then developed with DAB.

have the characteristic smear at the top of the gel that indicates the presence of high MW CPS. The smear was probably due to the heterogeneity of the polysaccharides (different numbers of repeating units). K31 and K3 CPS extracts were used as examples. K31 CPS extract contained the typical high MW smear at the top of the gel and then many bands close together. K3 also had the high MW smearing but low molecular weight bands were fewer and less frequent. For western blotting, K31 and K3 CPS extracts were transferred from a 10% gel onto a Nytran membrane and were detected with specific rabbit anti-capsular antiserum to K31 and K3. The K31 blot shows a strong positive signal at the top of the gel where high MW CPS were located, whereas K3 blot shows a weak signal across the whole length of the blot. This pattern was likely due to the degradation of the K3 CPS. The lower MW bands on the gel were not seen on the western blot. The evidence presented below suggests that these bands were not due to degraded CPS but were probably due to contaminating protein and LPS in the CPS extracts.

The K55 CPS extracts were fractionated by gel filtration chromatography with either dissociating buffer or PBS and then analysed by 10% (Figure 3.8) and 15% (Figure 3.9) SDS-PAGE. LPS was visualised by silver staining, and CPS was visualised by staining with Alcian blue followed by silver staining. *K. pneumoniae* CPS was not detectable without the use of alcian blue. Lanes 8 and 9 in Figure 3.8 (detection with alcian blue and silver staining) represent fractions that were eluted from the column with PBS. Both samples look very similar with a high MW smear and many low MW bands. The CPS extracts that were eluted with dissociating buffers (lanes 2-7), show very different

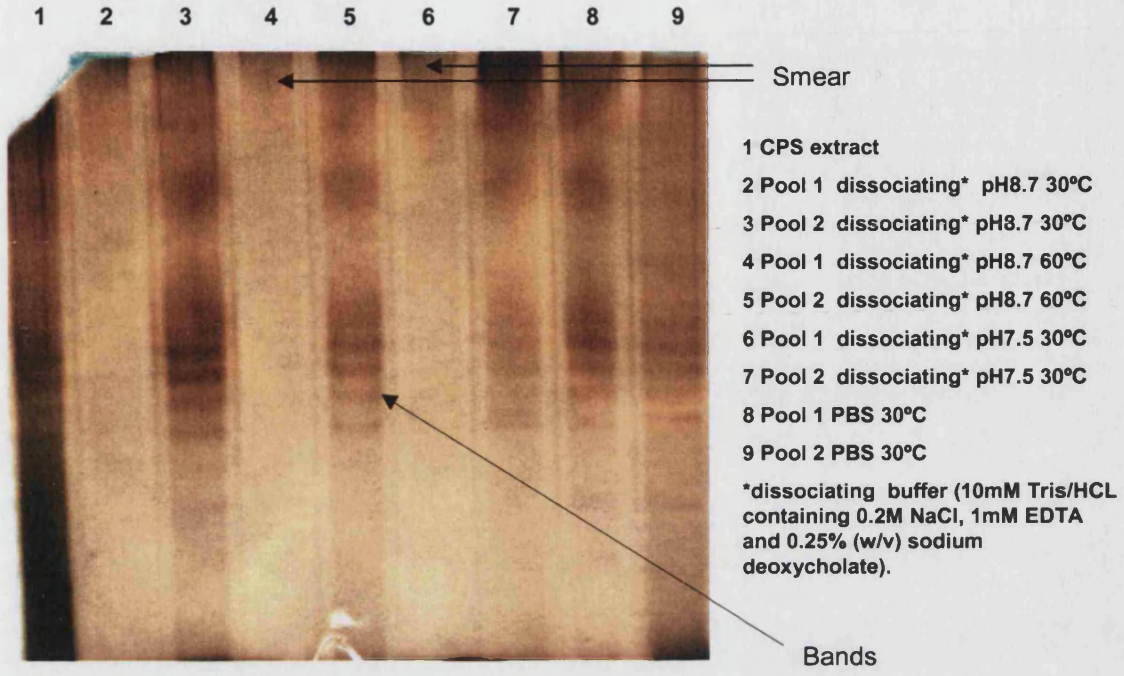


Figure 3.8 10 % SDS-PAGE analysis of *Klebsiella pneumoniae* capsular polysaccharide serotype K55 after gel filtration chromatography.

Chromatography was carried out in a TSK G5000 column with either PBS at 30°C or a dissociating buffer [10mM Tris-HCl (pH8.7 or 7.5) containing 0.2M NaCl, 1mM EDTA and 0.25% (w/v) sodium deoxycholate] at 30°C or 60°C. Peaks 1 and 2 containing carbohydrate were pooled and analysed by SDS-PAGE. Separation was carried out on a 10% gel and detection with Alcian blue followed by silver staining.

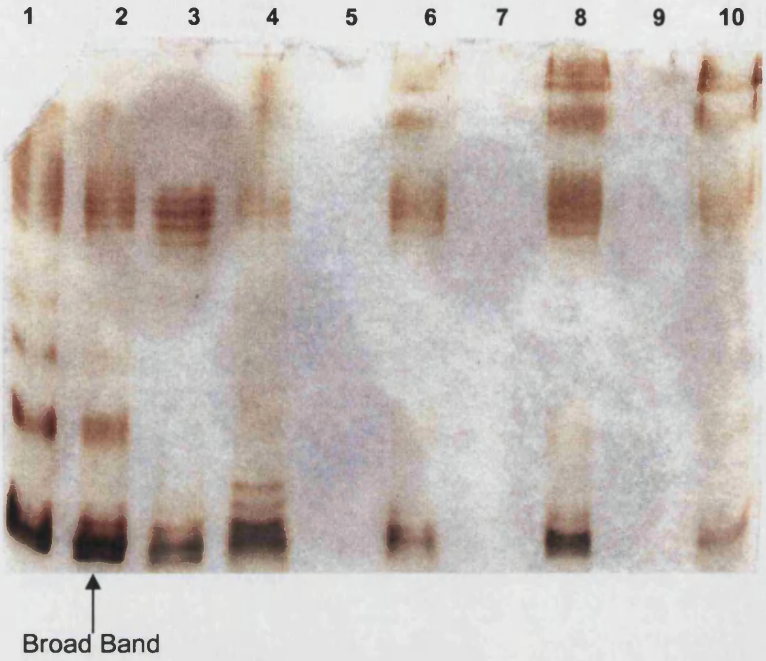


Figure 3.9 15% SDS-PAGE analysis of *Klebsiella pneumoniae* CPS serotype K55 after gel filtration chromatography

K55 CPS was extracted and purified by gel filtration chromatography. Chromatography was carried out on a TSK G5000 column with either PBS at 30°C or a dissociating buffer [10mM Tris-HCl (pH8.7 or 7.5) containing 0.2M NaCl, 1mM EDTA and 0.25% (w/v) sodium deoxycholate] at 30°C or 60°C. Peaks containing carbohydrate were pooled. CPS was then analysed along with LPS (extracted as shown in methods) by SDS-PAGE. Separation was carried out on a 15% gel and detection with silver staining.

profiles. Pool 1, which contained most of the CPS, had a characteristic smear at the top of the gel and no banding. The second pool had a smear and extensive banding which was due to LPS. Figure 3.9 (detection with silver staining only) shows a 15% SDS-PAGE of preparative run fractions. The gel was used to determine whether the bands seen in pool 2 on the 10% gel were due to CPS or LPS. The pools were compared to K55 LPS. Lanes 1 and 2 contained K55 LPS, and showed distinct banding pattern with a broad band at the bottom of the gel. The CPS eluted with PBS (lanes 3 and 4) gave very similar profiles to each other and to K55 LPS. Lanes 5-10 showed pools that were eluted from the gel filtration column with dissociating buffers. These all produced similar profiles to each other. Pool 1 showed very little or no staining at all whereas pool 2 showed similar staining to that seen for K55 LPS. This confirmed that pool 2 contained most if not all of the LPS, and pool 1 contained CPS. This was also consistent with the results of the LAL assay for pools 1 and 2.

In order to demonstrate that the banding patterns in pool 2 (high MW LPS components) seen on SDS-PAGE were not just specific for *K. pneumoniae* capsular serotype K55, K1 (Figure 3.10) and K52 (Figure 3.11 A) were also separated by gel filtration chromatography with PBS containing 0.25% (w/v) sodium deoxycholate. The carbohydrate-containing peaks were obtained and analysed by SDS-PAGE. In both cases, pool 2 contained material showing characteristic LPS profiles by SDS-PAGE. This includes the presence of one or more low MW bands. K55 and K1 have smooth-type LPSs whereas K52 is rough-type and therefore lacks the high molecular weight banding pattern.

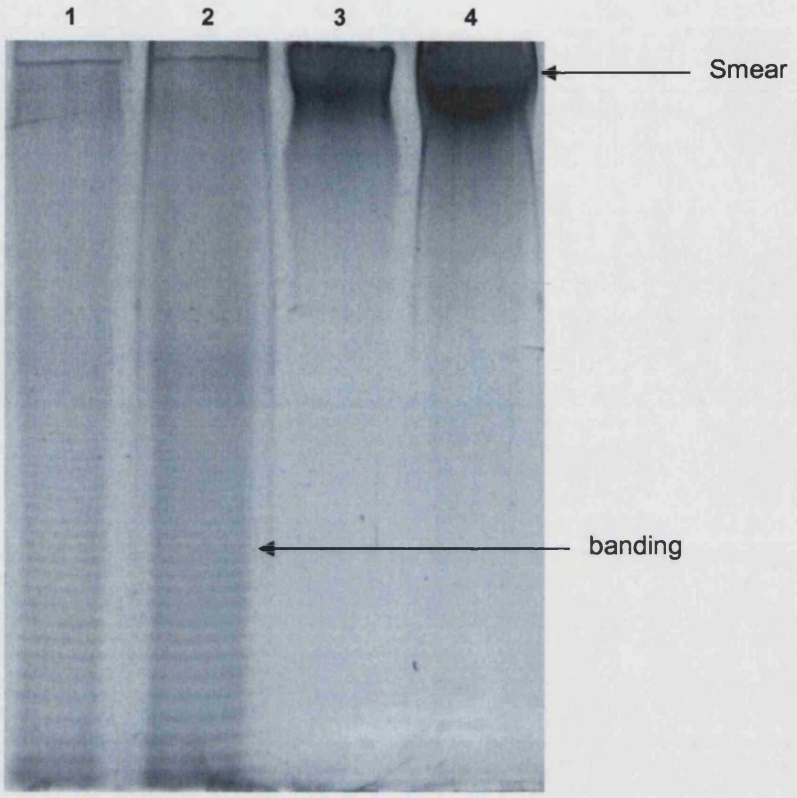


Figure 3.10 SDS-PAGE analysis of *Klebsiella pneumoniae* CPS serotype K3 pools 1 and 2.

Chromatography was carried out on a TSK G5000 column with PBS containing 0.25% (w/v) sodium deoxycholate at 60°C. Pools 1 and 2 were then analysed by SDS-PAGE. Separation was on a 10% (w/v) gel and detection with Alcian blue followed by silver staining. Lanes 1 and 2 = pool 2, Lanes 3 and 4 = pool 1.

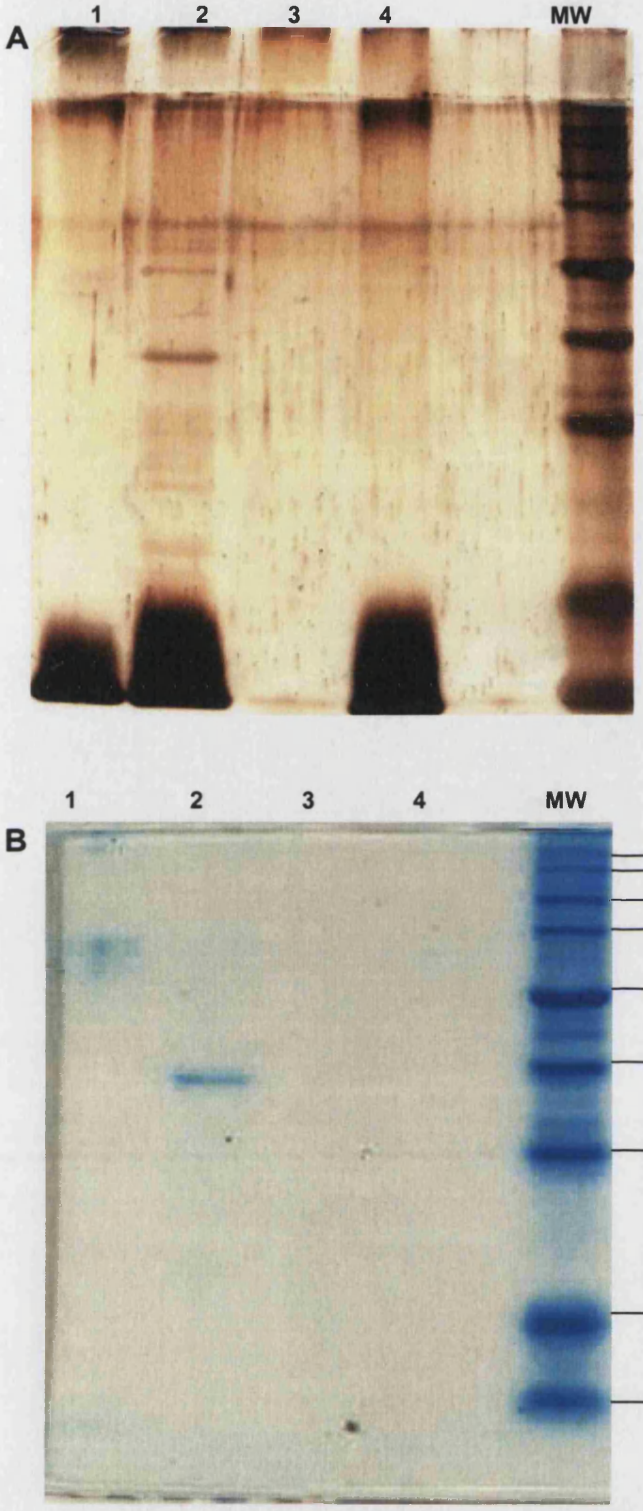


Figure 3.11 SDS-PAGE analysis of *Klebsiella pneumoniae* CPS serotype K52.

K. pneumoniae CPS was extracted as described in Section 2.2.1. Lane 1, K52 CPS extract (with protease digestion); Lane 2, K52 CPS extract (no protease digestion). After extraction CPS was purified by gel filtration chromatography on a TSK G5000 column in PBS containing 0.25% (w/v) sodium deoxycholate at 60°C. Peaks containing carbohydrate were pooled. Lane 3, K52 CPS, pool 1; Lane 4, K52 CPS, pool 2. Separation was on a 15% gel and detection with (A) silver staining or (B) Coomasie blue.

We have demonstrated that gel filtration chromatography with dissociating buffers was a good reproducible method for the purification of CPS from many of the *Klebsiella* capsular serotypes.

The effect of protease digestion on CPS extracts could also be followed by SDS-PAGE analysis. Figure 3.11 shows a comparison of K52 CPS extract with or without protease treatment (lanes 1 and 2). Both the Coomassie blue and silver staining detection methods show the disappearance of a number of bands in lane 1 compared to lane 2 after protease digestion of the samples.

3.5 Characterisation and purification of *Klebsiella pneumoniae* LPS.

3.5.1 Purification of *Klebsiella pneumoniae* LPS.

LPS from a number of different serotypes of *K. pneumoniae* were extracted by 45% (w/v) phenol extraction and purified by gel filtration chromatography as described in Section 2.2.2a. Chromatography was carried out on a HiPrep 26/60 Sephacryl S-300HR column in Tris-HCl, pH8.5, 20mM, 2mM EDTA and 1% (w/v) sodium deoxycholate at RT. Figure 3.12 shows a gel filtration profile of *K. pneumoniae* serotype K55 LPS. The LPS, as shown by refractive index trace, was found in two peaks at 260 and 300 min (indicated as A and B, respectively). Peak A contains full length LPS (O-antigen, core antigen and lipid) whereas peak B contains LPS without O-antigen. This was confirmed by SDS-PAGE analysis (Figure 3.13). Peak A containing full length LPS was pooled and used in experiments described in following chapters. Absorbance readings at 260nm and 280nm show that there was very little

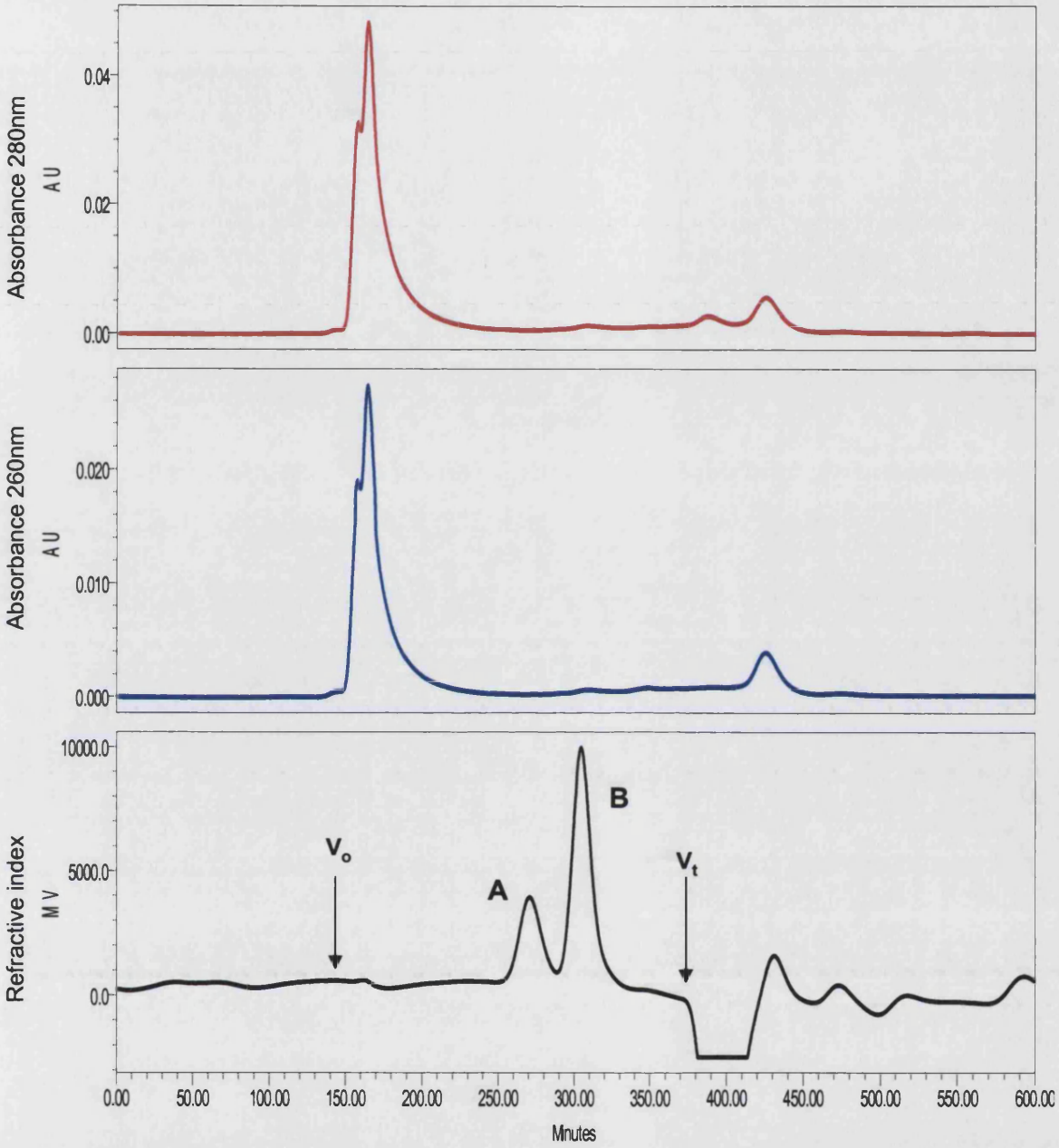


Figure 3.12 Gel filtration profile of *Klebsiella pneumoniae* serotype K55 LPS (HiPrep 26/60 Sephacryl S-300HR column)

K. pneumoniae LPS was extracted by aqueous 45% (w/v) phenol extraction and purified by gel filtration chromatography as described in Section 2.2.2. Chromatography was carried out on a HiPrep 26/60 Sephacryl S-300HR column in 20mM Tris-HCl, pH8.5, 2mM EDTA and 1% (w/v) sodium deoxycholate at room temperature. The flow rate was 0.6ml/min. The refractive index and absorbance (280nm and 260nm) of the eluant were monitored.

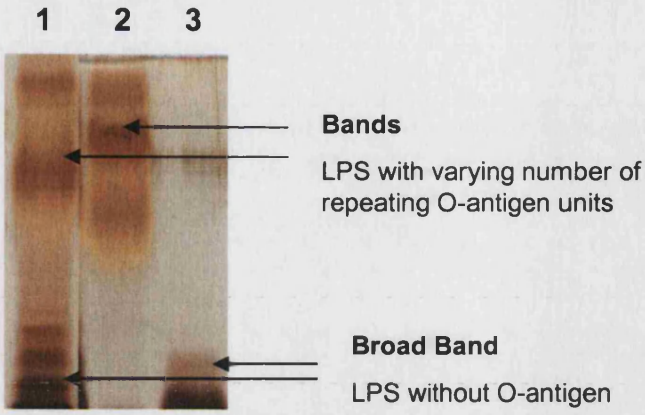


Figure 3.13 SDS-PAGE analysis of K55 LPS before and after gel filtration.

K. pneumoniae LPS was extracted by aqueous 45% (w/v) phenol extraction and purified by gel filtration chromatography as described in Section 2.2.2. The two LPS peaks as shown in Figure 3.12, A and B (Lane 2 and 3 respectively), were pooled separately and then analysed by SDS-PAGE on a 15% gel. Lane 1 represents LPS that was not subjected to gel filtration chromatography.

protein or DNA eluting at the same time as LPS. This was therefore a good method for removing DNA and protein contamination from LPS preparations. The possibility of CPS contamination in LPS preparations was also unlikely because during purification the sample was subjected to a 100,000 x g spin (Section 2.2.2a) that would have pelleted LPS but not CPS.

3.5.2 SDS-PAGE analysis of LPS from different O antigenic types of *Klebsiella pneumoniae.*

LPS was extracted from different O-antigenic *Klebsiella* strains, R:K17, O4:K3, O2:K3 and O1:K1, which represent each of the LPS types [polymannose O-antigen (O3), polygalactose (O1 and O2), ribose/galactose O-antigen (O4) and rough type LPS (R)]. LPS was then characterised by SDS-PAGE as shown in Figure 3.14. The banding patterns were different for each LPS studied, although they all have the broad intense band at the bottom of the gel that represents LPS with no O-antigen. The high MW bands in lanes containing K36, K3 and K1 are due to LPS with different numbers of repeating units of O-antigen. K17 which has a rough LPS (i.e. no O-antigen) shows no high MW banding (Lane 1) when a low concentration was loaded onto gel. When high concentration of K17 LPS was loaded onto the gel (Lane 2) high MW bands were observed. This was probably due to LPS micelle formation.

3.6 Discussion

The aim of the work described in this chapter was to improve methods of CPS purification in order to obtain highly purified *K. pneumoniae* CPS preparations that could be used to investigate their immunogenicity and immunomodulating properties. It was very important that sample preparations

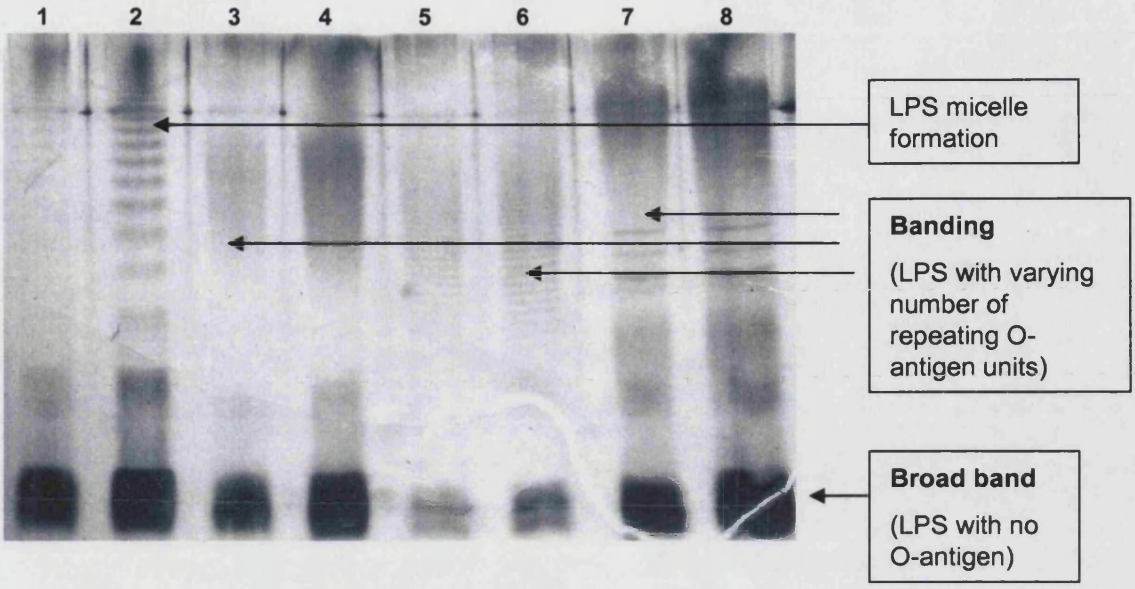


Figure 3.14 SDS-PAGE analysis of LPS from various *Klebsiella pneumoniae* capsular serotypes.

K. pneumoniae LPS was extracted by aqueous 45% (w/v) phenol extraction. The LPS was then analysed by SDS-PAGE on a 15% gel. LPS was visualised by silver staining. Lanes 1 and 2, LPS extracted from capsular serotype strain K17, 0.2μg and 0.4μg; Lanes 3 and 4 LPS from serotype K36, 0.2μg and 0.4μg; Lanes 5 and 6 LPS from serotype K3, 0.2μg and 0.4μg; Lanes 7 and 8 LPS from serotype K1, 0.2μg and 0.4μg.

used were free from contaminants, in particular those that have a major influence on the immune system. When extracting CPS from *K. pneumoniae* many other components are co-extracted, including LPS, proteins, nucleic acids and lipoproteins. All of these microbial products are recognised by toll-like receptors and have been reported to result in an inflammatory response. The presence of even small amounts of endotoxin in samples could cause adverse reactions such as shock when these preparations were used as antigens for immunising experimental animals (Fraker et al., 1988). It is therefore critical that LPS is removed from preparations.

Classical purification and chemical detoxification methods have been used and evaluated by others for their effectiveness in removing residual LPS, whilst still preserving the structural and functional integrity of the CPS. Previous studies indicated that LPS could not be removed from CPS by ultracentrifugation, anion-exchange chromatography or gel filtration chromatography with acetic acid (Adam et al., 1995). The use of chemical treatments like de-O-acylation can be an effective way to detoxify the LPS (Seid and Sadoff, 1981), but similar treatments can lead to loss of structure or immunogenicity of the CPS depending on polysaccharide structure. Using a relatively high pH can lead to the loss of alkali labile substituents such as O-acetyl groups and probably partial de-O-acylation of lipids (CPS-lipid anchors). For examples, the serogroup A of *N. meningitidis* CPS contains O-acetyl groups which can be easily removed at an alkaline pH and results in its reduced immunogenicity (Berry et al., 2002). Some of the *Klebsiella* CPSs contain O-acetyl groups: these include K55 (Kenne and Lindberg,

1983) and K22 (Parolis et al., 1988) so their structure may be affected by alkaline detoxification. A method described by Adam and colleagues using gel filtration chromatography with a sodium deoxycholate containing buffer (Adam et al., 1995) showed promise of being a good method for CPS purification as very little LPS remained in the preparation. The only problem anticipated with this method was the high pH and temperature of the buffer and its possible effect on the CPS immunogenicity and structure. It was therefore investigated further and modification of conditions was considered.

CPS extracts were first fractionated on a TSK G5000 gel filtration column without detergent. The carbohydrate eluted in two peaks as shown by refractive index profiles. The first high MW peak contained 90% of the carbohydrate recovered from the column. Following ultracentrifugation and protease and nuclease digestion of CPS extracts, the protein and nucleic acids contaminants eluted later from the column than the high MW CPS (Figure 3.3). This allowed CPS to be pooled that contained less than 1% protein and no nucleic acid detectable by UV absorbance.

Removal of LPS was much more difficult. A method described using Triton-X114 failed to remove a significant amount of LPS. More successful was the use of gel filtration chromatography with dissociating buffers. CPS extracts were fractionated on a TSK G5000 column with a range of dissociating buffers. Two carbohydrate peaks were obtained. When dissociating buffers were used, LPS micelle formation was disrupted and therefore resulted in LPS eluting later from the column than CPS (Figure 3.4). Therefore CPS

could be pooled and separated from LPS. Subsequent SDS-PAGE analysis confirmed the presence of LPS in the CPS extracts. Pool 2 (lower MW carbohydrate) from the gel filtration of K55 separated under dissociating conditions gave similar profiles to those of K55 LPS (Figure 3.9). In contrast, pool 1 (high MW carbohydrate) appeared to be LPS free. CPS could not be detected in pool 1 with silver staining only (Figure 3.9, lanes 5, 7, and 9), but with a combination of Alcian Blue and silver staining CPS was detected (Figure 3.8, lanes 3, 4 and 6). Pool 1 therefore contained highly purified CPS.

Several dissociating buffers were investigated and all gave similar refractive index profiles which indicated no gross change in CPS MW. Recovery was also very similar for all conditions at 80% or greater. The only variation was the effect on the antigenicity of the polysaccharide. Some of the buffers were of high pH or used at high temperatures, and these could affect CPS structure and its antigenicity. Antigenicity was determined by the binding of rabbit anti-capsular antiserum to pool 1 compared to control CPS (not subjected to gel filtration chromatography). When the CPS extract was eluted with PBS containing 0.25% (w/v) sodium deoxycholate at 60°C there was no change in antibody binding to the CPS. It was for this reason that this elution buffer was used for CPS purification, although it must be noted that these antigencity experiments were only carried out for K55 CPS.

In conclusion, previous methods of CPS purification have been modified. These modifications have been shown to have no effect on CPS immunogenicity and molecular weight. Gel filtration profiles, SDS-PAGE

analysis and quantification methods have shown that pool 1 (high MW CPS) is free from nucleic acid and contains no more than 1% protein and 0.01% LPS by weight. Although 0.01% LPS may still have biological effects, LPS hyporesponsive mice will be used in experiments in Chapters 4 and 5 to determine the properties of CPS independent of LPS. The CPS preparation therefore could confidently be used for our immunological studies.

Chapter Four

Results

Chapter Four

The immunogenicity and adjuvanticity of capsular polysaccharides from *Klebsiella pneumoniae.*

4.1 Introduction

K. pneumoniae is a Gram negative bacterium in the genus *Klebsiella* of the family Enterobacteriaceae. It is an opportunistic pathogen that frequently causes nosocomial infections, mainly in immunocompromised patients. Infections range from mild urinary infections to severe bacteremia and pneumonia with a high mortality and morbidity rate. *K. pneumoniae* is encapsulated and antibodies toward the CPS are protective (Donta et al., 1996). Antigenic capsule material is termed the K antigen, and there are at least 90 K antigens reported. The structures of most of the K antigens have been determined. They typically contain one charged monosaccharide constituent, normally glucuronic acid, together with hexoses and 6-deoxyhexoses. In addition, non-carbohydrate constituents, such as formyl or acetyl groups and ketal-linked pyruvates are also found (Orskov, 1984).

K. pneumoniae LPS itself is of interest. Chemically, LPS consists of an O-specific polysaccharide sidechain, a core oligosaccharide, and a lipid component, termed lipid A. Structural differences in O-polysaccharide have been exploited for serological typing based on O-antigens (Kelly et al., 1993). In contrast to the large number of capsular serotypes (K antigens), only 9 O-antigens have been recognised in *K. pneumoniae* based on structure and

reactivity of the O-polysaccharides with specific antibodies and structure (Hansen et al., 1999). Serotype 01 is the most common among clinical isolates. The remaining eight groups are made up of 02, 02ac, 03, 04, 05, 07, 08, and 012. The O antigens consist of repeating units containing different monosaccharides, such as D-Man, D-Gal and L-Rha and in some cases substituted with O-acetyl groups.

Several reports in the literature describe strong immunostimulatory, anti-tumour and adjuvant effect for capsular polysaccharide extracts from *K. pneumoniae.* The CPS extracts have been shown to exhibit strong adjuvant effects on antibody responses to various antigens in mice (Nakashima et al., 1971; Nakashima, 1972; Nakashima and Kato, 1975). After mice were immunised subcutaneously with CPS the number of cells in lymphoid organs increased (Yokochi et al., 1980a). *K. pneumoniae* CPS K1 and K3 in particular were shown to induce the release of TNFα, and they could also suppress tumours in mice (Ho et al., 2000; Choy et al., 1996). The exact composition of the extracts and the nature of the immunostimulatory components are not known. In some cases these activities have claimed to be attributed directly to CPS, and not, for example, to contaminating LPS in the preparations.

Commercially, a *K. pneumoniae* polysaccharide extract, Biostim, is used to boost the immune system. Biostim, also known as RU-41740 or C1740, is a cell wall extract consisting of at least two large glycoproteins. When administered orally, Biostim has been shown to boost the immune system

against chronic bronchial bacterial infections (Fietta et al., 1992) and to enhance antibody responses to intramuscularly administered inactivated influenza virus vaccine (Profeta et al., 1987). *In vitro* studies with LPS-hyporesponsive mouse B cells (Wood and Moller, 1984) and human B cells (Martinez-Maza et al., 1985) have shown Biostim to be a polyclonal B cell activator. Biostim is a very heterogeneous mixture, and the actual component (s), with polyclonal B cell activation ability has not yet been reported.

Certain O-polysaccharide structures of *K. pneumoniae* LPS appear to have greater adjuvant properties than others. The mannose homopolymers present in *K. pneumoniae* O3 and O5 have been shown to induce greater antibody responses in mice to soluble protein antigens than LPS with other O-polysaccharide structures (Ohta et al., 1985; Kido et al., 1985). An adjuvant effect was also observed in mice which are hyporesponsive to LPS (Ohta et al., 1985). Mutants that lack O-specific polysaccharide derived from *K. pneumoniae* 03 had weaker adjuvant activity than the parental LPS (Ohta et al., 1987) suggesting that the mannose-rich O-polysaccharide is essential for adjuvant activity. Yokochi and colleagues showed that *K. pneumoniae* 03 LPS could activate the complement pathway to a greater extent than non mannose containing O-polysaccharide LPSs, although the mannose-rich O-polysaccharide in the absence of lipid A did not stimulate complement (Yokochi et al., 1990). Paeng and colleagues (1996) have produced an *E. coli* LPS that possesses a mannose homopolymer by transforming *E. coli* K-12 with *rfb* genes that are required for synthesising the mannose

homopolymer on LPS. Similar to *K. pneumoniae* 03 LPS, it has the ability to activate complement, and also to cause enlargement of regional lymph node.

In summary, both LPS and CPS from *K. pneumoniae* have been shown to have immunostimulatory properties. LPS expressing a mannose-rich O-polysaccharide has enhanced adjuvant activity compared to other LPS and activates complement to a greater extent. CPS extracts have the ability to enhance antibody production and have anti-tumour effects. Investigating the immunomodulating properties of *K. pneumoniae* LPS and CPS would provide important insights into the mechanisms of activation of innate immunity by microbial carbohydrates.

4.2 Objectives

The aim of the work described in this chapter was to carry out further investigations on the adjuvanticity of *K. pneumoniae* CPS extracts, and to establish, in particular, whether any biological activity could be attributed to the CPS itself.

4.3 The adjuvant effect of *Klebsiella pneumoniae* CPS and LPS extracts on the humoral response to CGG in mice

To assess the adjuvant activity of CPS and LPS extracts from *K. pneumoniae* the IgG response to chicken gamma globulin (CGG) in the presence of these components was determined. Only IgG response to CGG was measured because previous studies have shown that the antibody response generated to CGG is predominantly IgG, and the presence of an adjuvant is an absolute

requirement for antibody production (Le Bon et al., 2001). The CPS extracts used in this study have been subjected to ultracentrifugation and protease and nuclease digestion unless otherwise stated. The typical content by weight of a CPS extract was 10–15% protein, 5% nucleic acid, and 1-2% LPS. For all experiments, unless stated otherwise, groups of 3 mice were immunised by the intra peritoneal route with 5μg of CPS extract or LPS together with 100μg of CGG. Mice were bled 7 and 21 days post immunisation and serum was collected. The serum was pooled for each group of mice due to the large number of CPS preparations to be tested. The antibody response to CGG was assessed by ELISA as described in Section 2.2.4g.

4.3.1 Adjuvant properties of *K. pneumoniae* CPS and LPS in C57BL/6 mice

In order to confirm the adjuvant potential of *K. pneumoniae* CPS and LPS studies were performed in comparison with a known adjuvant, Titermax. Preliminary studies were carried out in C57BL/6 mice. Figure 4.1 shows the antibody response to CGG using *K. pneumoniae* K1 and K3 CPS and LPSs from capsular serotypes K1/K3 (combined) and K55 as adjuvants. These LPS types were used because K1 and K3 O-polysaccharides are homopolymers of D-Gal and K55 O-polysaccharide of its LPS is a mannose homopolymer. At days 7 and 21 an increase in IgG to CGG was observed for all adjuvants compared to mice immunised with only CGG. K1/K3 CPS extract and K55 LPS enhanced the IgG response to CGG to a greater extent than other adjuvants at day 7. At day 21 K55 LPS induced the greatest

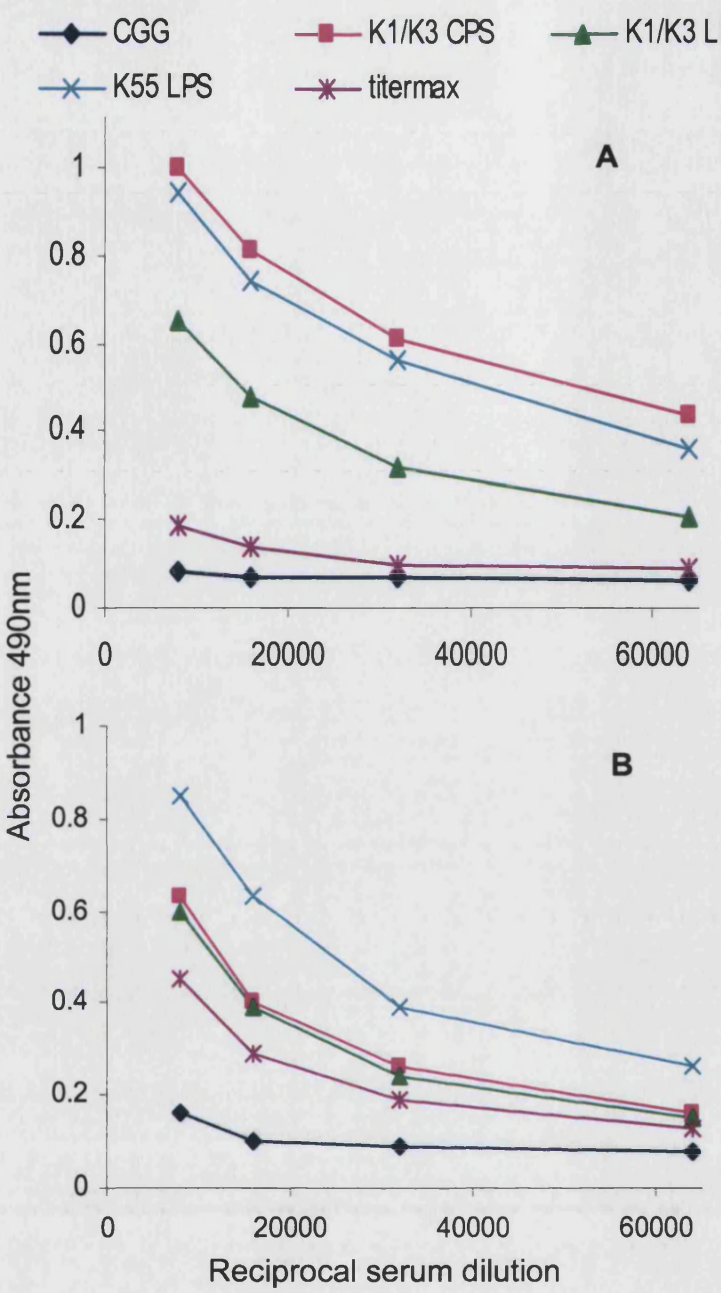


Figure 4.1 The effect of different adjuvants on the serum IgG antibody response to chicken gamma globulin (CGG) in C57BL/6 mice

Groups of three C57BL/6 mice were immunised by the intra peritoneal route with 100μg of CGG in PBS with or without adjuvant. When injected with Titermax, an equal volume of CGG was emulsified with the adjuvant before injection. When injected with LPS and CPS, 5μg of adjuvant was mixed with the CGG. The mice were bled at day 7 (A) and 21 (B), serum was pooled for each group and then analysed for IgG by ELISA. IgG was detected using goat anti-mouse IgG (γ chain specific) HRP conjugate and the reaction developed using OPD substrate. Absorbance was read at 490nm. These data are means of duplicate wells and are from one representative study out of two.

adjuvant effect. At day 7 and 21 Titermax induced the lowest levels of IgG to CGG compared to other adjuvants.

4.3.2 Adjuvant properties of *K. pneumoniae* CPS and LPS in C3H/HeJ and C3H/HeN mice.

Although preliminary studies showed that *K. pneumoniae* K1 and K3 CPS extracts could induce an antibody response to CGG, the possibility that the adjuvant effect was due to contaminating LPS could not be ruled out. To control for LPS contribution to adjuvanticity C3H/HeJ mice were used. These mice are hyporesponsive to LPS.

The presence of small amounts of contaminating LPS in commercial stocks of CGG was confirmed by the LAL assay. To determine whether this contributed to the immunogenicity of CGG, the responses to CGG before and after LPS removal were compared in C3H/HeJ and C3H/HeN (wild type) mice (Figure 4.2). Clearly without LPS removal there was a response to CGG in C3H/HeN but not C3H/HeJ mice, confirming that the CGG contained LPS which acts as an adjuvant in C3H/HeN mice. When LPS is removed no IgG could be detected in either C3H/HeN or C3H/HeJ mice. This enforces the idea that an adjuvant was required to induce an antibody response to CGG.

The adjuvant activity of *K. pneumoniae* K1/K3 and K55 CPS extracts and K55 and K1/K3 LPS were studied in C3H/HeJ mice (Figure 4.3). The reported immunostimulatory properties of *K. pneumoniae* CPS extracts have been limited to mainly those from K1 and K3 (Ho et al., 2000; Choy et al., 1996), so another CPS was included to determine whether the activity

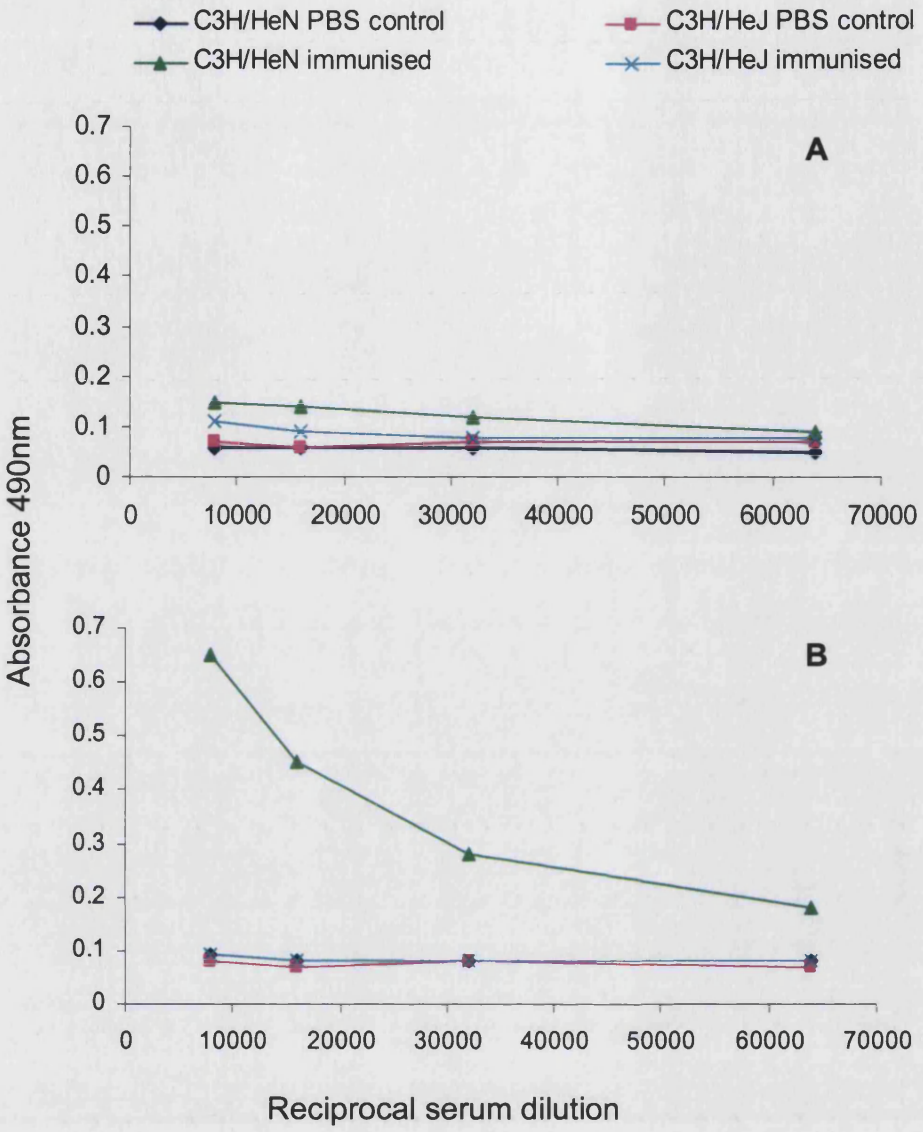


Figure 4.2 A comparison of serum IgG antibody responses in C3H/HeN and C3H/HeJ mice after LPS removal from chicken gamma globulin (CGG).

LPS was removed from CGG using a Vivapure Mini Q spin column. Groups of 3 C3H/HeN and C3H/HeJ mice were immunised by the intra peritoneal route with 100μg of CGG with (A) or without (B) LPS removal. Mice were bled at day 7 and serum was pooled and assessed by ELISA as described in Section 2.2.4g. These data are means of duplicate wells and are from one representative study out of two.

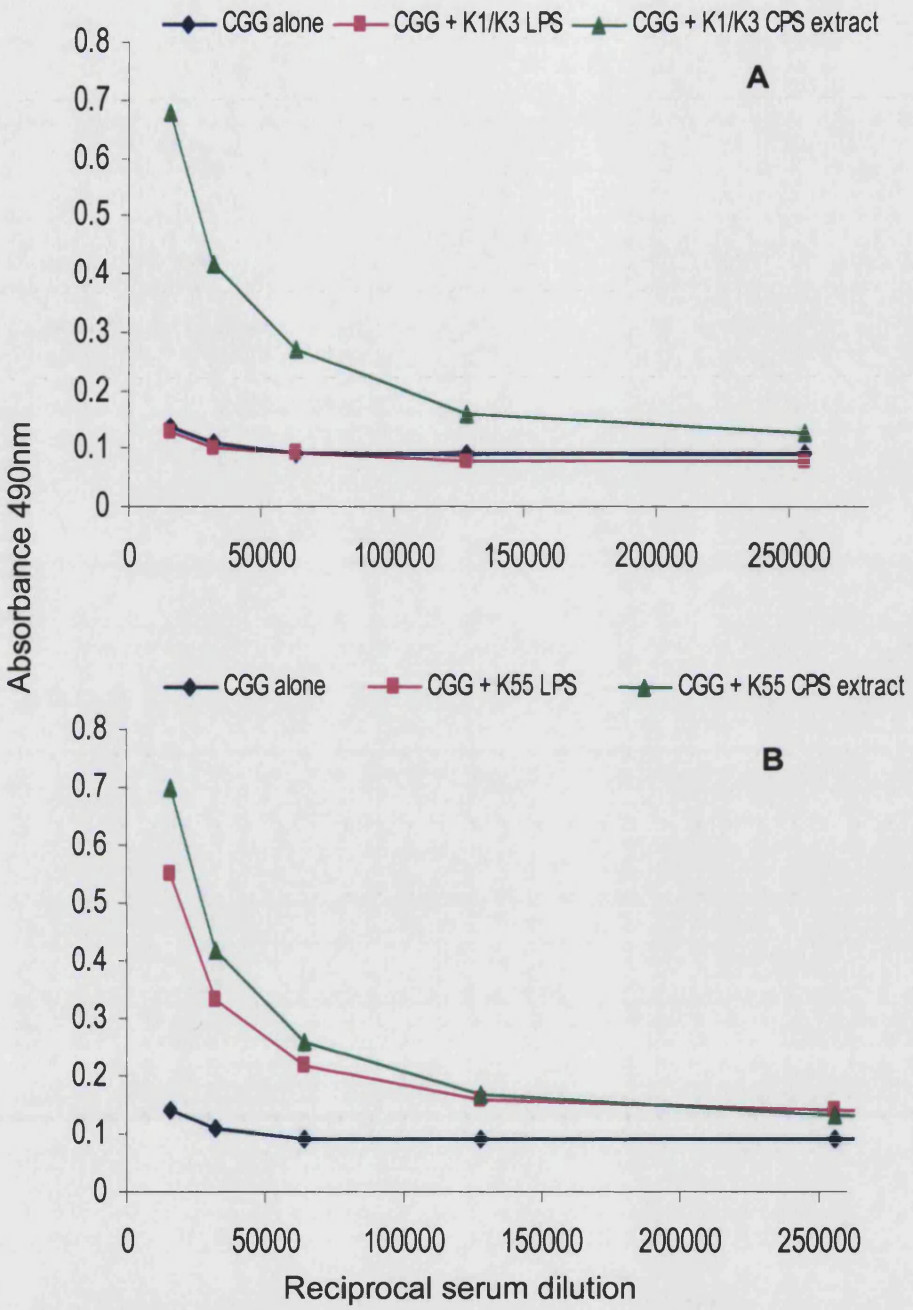


Figure 4.3 A comparison of the adjuvanticity of CPS and LPS from *K. pneumoniae* K1, K3 and K55 in C3H/HeJ mice

Groups of three C3H/HeJ mice were immunised by the intra peritoneal route with 100μg of chicken gamma globulin (CGG) mixed with 5μg of LPS or CPS extract from K1/K3 (A) or K55 (B). Mice were bled at 21 days and serum was pooled and assessed for IgG to CGG by ELISA. IgG was detected by goat anti-mouse IgG (γ chain specific) HRP conjugate and developed with OPD substrate. Absorbance readings were taken at 490nm wavelength. These data are means of duplicate wells and from are one representative study out of two (A) or four (B).

reported was unique to those strains. In this study K1, K3 and K55 CPS extracts and K55 LPS were shown to act as adjuvants in C3H/HeJ mice. The IgG levels with the inclusion of CPS or LPS were much greater than with CGG alone. There was no IgG response to CGG using K1 and K3 LPS as adjuvants in C3H/HeJ mice. Interestingly, K55 LPS still functioned as an adjuvant in C3H/HeJ mice.

The effect of dose of CPS extract on the IgG response to CGG was also investigated (Figure 4.4). *K. pneumoniae* K55 CPS extract is shown as an example. In C3H/HeJ and C3H/HeN mice 50μg and 25μg doses elicited greater IgG levels to CGG than 5ug. It important to note that experiments were nevertheless carried out using 5ug of CPS extract as an adjuvant because the response was still easily detectable. It was also more practical to use as little CPS extract as possible due to the length of time required for extraction and purification.

Figure 4.5 shows the relative decrease in adjuvanticity of *K. pneumoniae* CPS and LPS extracts in C3H/HeJ compared to C3H/HeN. For K1 and K3 LPS extracts the adjuvant activity was completely abrogated in the C3H/HeJ mice, but remained in C3H/HeN mice. For K55, K1 and K3 CPS extracts the response was reduced in C3H/HeJ mice compared to C3H/HeN, which suggested that LPS contributes to the CPS extracts' adjuvanticity in C3H/HeN mice. Surprisingly there was a reduction in the response of mice to Titermax in C3H/HeJ mice, even though the Titermax was shown to be free from LPS by the LAL assay. These results illustrate the adjuvant properties

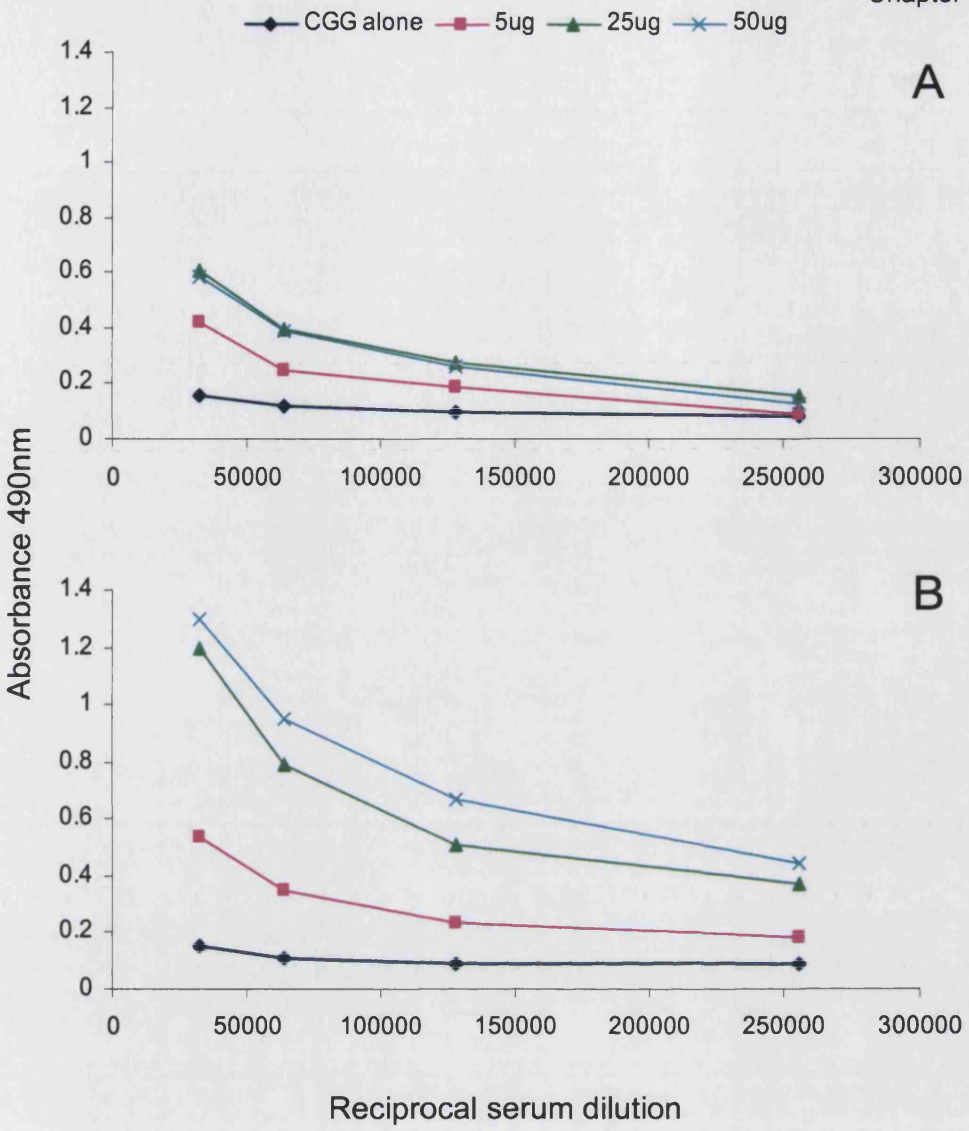


Figure 4.4 The effect of dose of *K. pneumoniae* K55 CPS extract on the IgG antibody response to chicken gamma globulin (CGG).

Groups of three C3H/HeN and C3H/HeJ mice were immunised by the intra peritoneal route with 100μg of CGG mixed with K55 CPS extract (5, 25 or 50μg). C3H/HeJ (A) and C3H/HeN (B) mice were bled at day 21 and serum antibody was pooled and then assessed by ELISA for IgG. These data shown are means of duplicate wells and were from one representative study out of two.

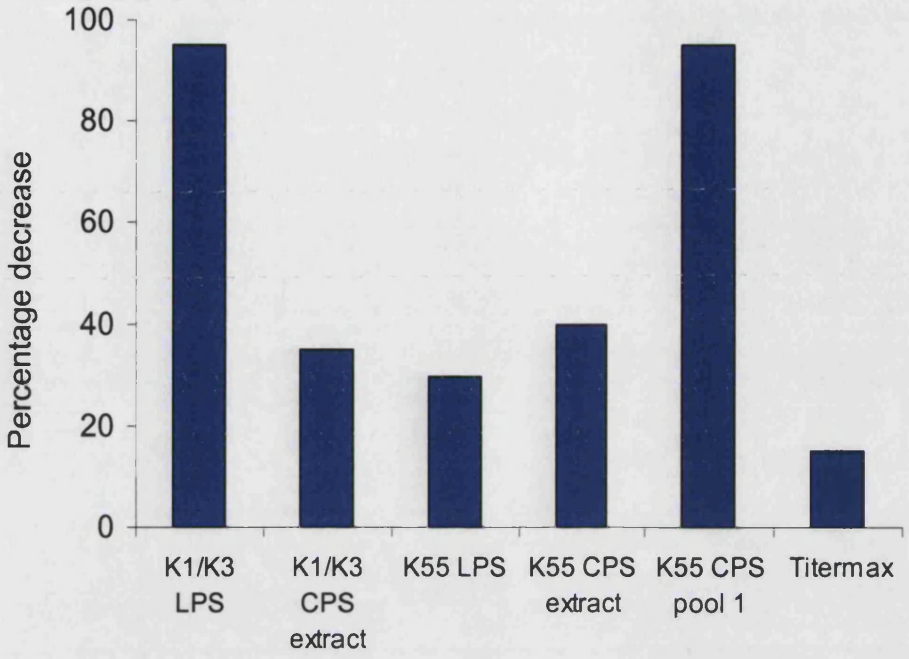


Figure 4.5 Relative decrease in adjuvanticity of *K. pneumoniae* CPS and LPS extracts in C3H/HeJ compared with C3H/HeN mice.

C3H/HeN and C3H/HeJ mice were immunised by the intra peritoneal route with 100μg chicken gamma globulin (CGG) mixed with 5μg of either K1/K3 LPS, K1/K3 CPS extract, K55 LPS, K55 CPS extract, K55 CPS pool 1 or Titermax. Mice were bled at day 21 and serum IgG antibody levels were assessed by ELISA. IgG responses were compared in the C3H/HeN and C3H/HeJ mice by calculating the antibody level (titre) in the C3H/HeJ mice as a percentage of the titre in C3H/HeN mice. Results are ± 10% taken as the average of three experiments.

of LPS but also the adjuvant activity of CPS extracts independent of LPS. Although adjuvant activity was still maintained in C3H/HeJ mice for CPS extracts, the magnitude of the response was reduced compared to IgG levels observed in C3H/HeN mice.

4.4 The contribution of the polysaccharide component of *Klebsiella pneumoniae* CPS extracts to adjuvanticity.

As described earlier, several studies have suggested that the adjuvanticity of *K. pneumoniae* CPS extracts is due to CPS and not other components in the preparations (Ho et al., 2000; Choy et al., 1996; Yokochi et al., 1980a; Nakashima, 1972; Nakashima and Kato, 1975; Nakashima et al., 1971). Methods for preparing CPS in these studies were not adequately described making it difficult to assess the purity of the CPS used. The purification methods used for this project are described in Chapter 3. Briefly, *K. pneumoniae* CPS were extracted and subjected to ultracentrifugation and protease and nuclease digestion. This sample was referred to as *K. pneumoniae* CPS extract, and was used for the studies described in this chapter. Further purification of CPS extracts was carried out. CPS extracts were separated by gel filtration on a TSK G5000 column with PBS containing (w/v) 0.25% sodium deoxycholate at 60°C and two carbohydrate peaks were obtained. Pool 1 contained most of the carbohydrate material so a CPS concentration could be determined. Mice were immunised with CGG containing 5μg of CPS pool 1. For pool 2 a volume equal to that which contained 5μg of CPS in pool 1 was used. The typical content by dry weight

of CPS pool 1 was 1% protein, 0-0.01% nucleic acid, 0.01% LPS. Pool 2 contained most of the LPS and protein from the CPS extract.

The adjuvant effect of CPS pools 1 and 2 from serotypes K17, K55, and K52 was investigated (Figures 4.6-4.8). Figure 4.6 shows the IgG response to CGG when K17 CPS pool 1 was used as an adjuvant in both C3H/HeJ and C3H/HeN mice. There was no IgG response to CGG in C3H/HeJ mice indicating that the high MW purified CPS pool 1 did not have adjuvant effects on antibody responses. An IgG response to CGG in C3H/HeN mice, however, was observed, which indicated that the adjuvant activity of the K17 CPS pool 1 was solely due to LPS. Figure 4.7 shows the adjuvant effects of K55 CPS pools 1 and 2, and the CPS extract on the IgG response to CGG in C3H/HeJ mice. Similar to K17 CPS pool 1, there was no adjuvant activity in K55 CPS pool 1. Most of the adjuvant activity was present in K55 CPS pool 2 and at a level equivalent to that of unfractionated K55 CPS extract. The same conclusions could be drawn from Figure 4.8. In C3H/HeJ mice there was no adjuvant effect of K52 CPS pool 1, and the response seen in C3H/HeN mice was therefore due to LPS. The adjuvant activity was contained within K52 CPS pool 2. These results suggest that the *K. pneumoniae* CPS pool 1, a very pure CPS preparation, from a number of different serotypes did not enhance the IgG response to CGG independent of LPS. Most of the adjuvant activity is attributed to *K. pneumoniae* CPS pool 2 as shown for K55 and K52.

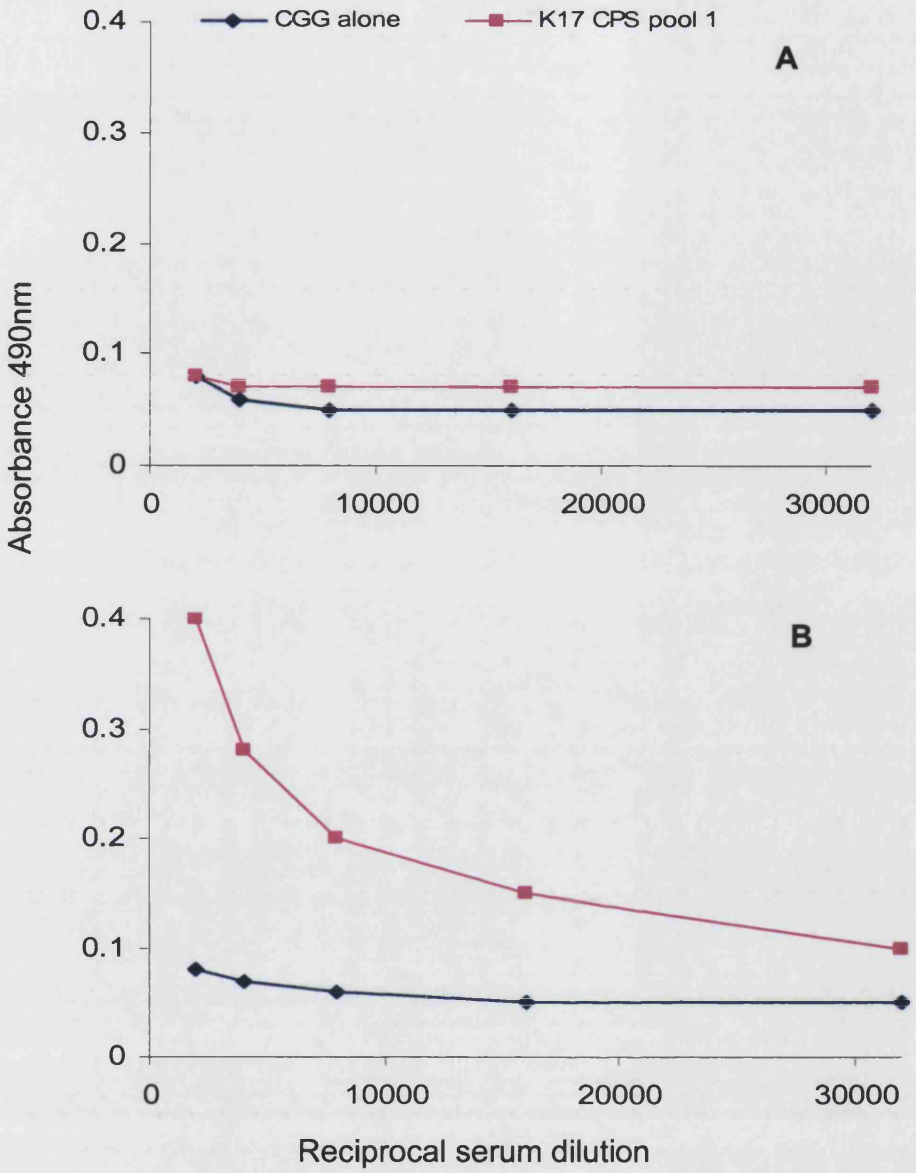


Figure 4.6 Serum IgG responses to chicken gamma globulin (CGG) using *K. pneumoniae* K17 CPS pool 1 as an adjuvant in C3H/HeJ and C3H/HeN mice

K17 CPS extract was separated by gel filtration chromatography on a TSK G5000 column with PBS containing 0.25% (w/v) sodium deoxycholate and refractive index-positive peaks 1 and 2 were pooled. Groups of three C3H/HeJ (A) and C3H/HeN (B) mice were immunised by the intra peritoneal route with 100μg CGG mixed with K17 pool 1 CPS (5μg). Mice were bled 21 days post immunisation. Blood serum was pooled and analysed by ELISA as described in Section 2.2.4g. The data shown are means of duplicate wells and are from one representative study out of three.

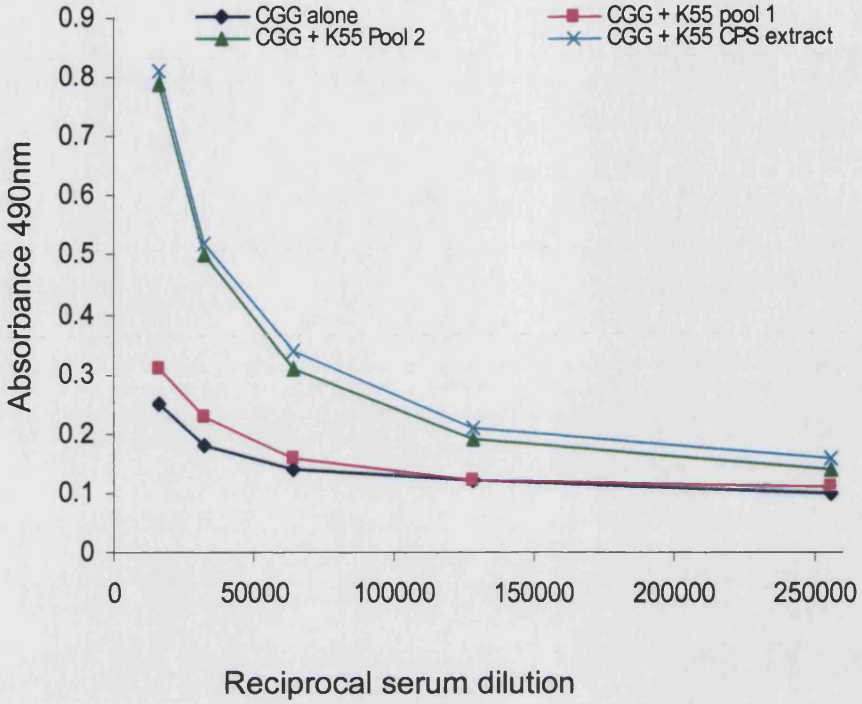


Figure 4.7 Serum IgG responses to CGG using *K. pneumoniae* K55 CPS pools 1 and 2 as adjuvants.

The CPS pools were collected after gel filtration chromatography of K55 CPS extract on a TSK G5000 column with PBS containing 0.25% (w/v). Groups of three C3H/HeJ and C3H/HeN (data not shown) mice were immunised by the intra peritoneal route with 100μg of CGG mixed with 5μg of either K55 CPS pool 1 or pool 2. Mice were bled 21 days post immunisation, serum was pooled and then analysed for IgG by ELISA, as described in Section 2.2.4g. These data shown are means of duplicate wells and are from one representative study out five.

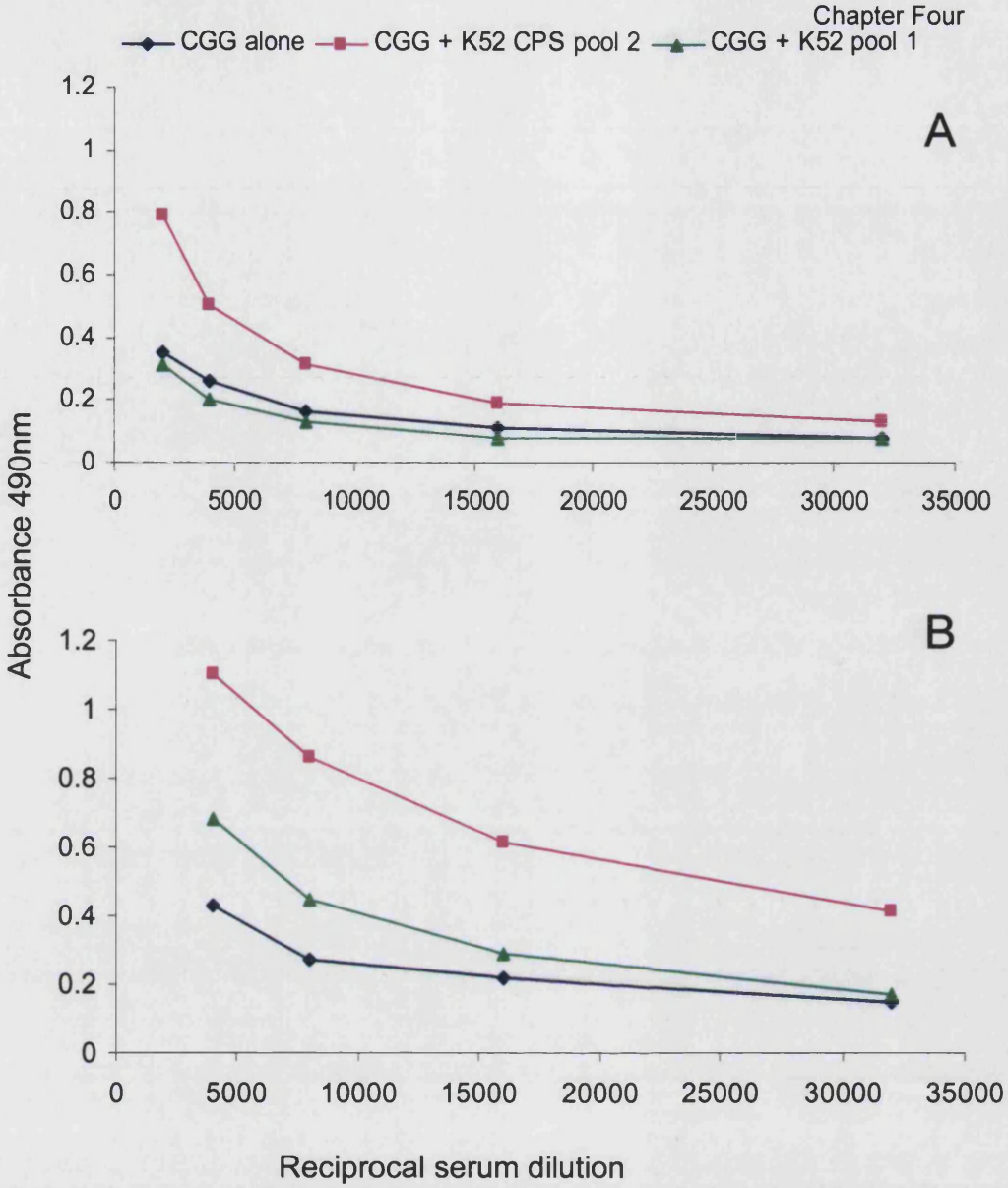


Figure 4.8 A comparison of serum IgG responses to chicken gamma globulin (CGG) using K52 CPS pool 1 and 2 as adjuvants in C3H/HeJ and C3H/HeN mice.

The CPS pools were collected after gel filtration chromatography of K52 CPS extract on a TSK G5000 column with PBS containing 0.25% (w/v) sodium deoxycholate. Groups of three C3H/HeJ (A) and C3H/HeN (B) mice were immunised by the intra peritoneal route with 100μg CGG mixed with either K52 CPS pool 1 (5μg CPS) or pool 2. Mice were bled 21 days post immunisation, serum was pooled and analysed by ELISA as described in Section 2.2.4g. The data shown are means of duplicate wells and are from one representative study out of two.

To further analyse the adjuvant properties of K55 CPS extract, the effect of protease treatment was investigated. Mice were immunised with CGG together with K55 CPS extract either before or after protease treatment to identify any difference in adjuvant properties of the samples. Results are shown in Figure 4.9. There was a reduction in the levels of IgG to CGG when the CPS extract was protease treated, but the adjuvant activity was still present.

The effect of varying degrees of alkali treatment upon the adjuvant properties of K55 CPS extract was investigated. K55 CPS extract was suspended in 0.1M NaOH and incubated for either 1h at room temperature, 1h at 37°C, or 18h at 37°C. Samples together with CGG were then used to immunise C3H/HeN and C3H/HeJ mice. The results are shown in Figure 4.10. The serum IgG levels to CGG are greatest in both C3H/HeN and C3H/HeJ when K55 CPS extract was used as an adjuvant. Following alkali treatment of the K55 CPS extract, the IgG levels to CGG decreased when temperature and incubation time for alkali treatment were increased. After alkali treatment for 18h at 37°C adjuvant properties were lost in both C3H/HeJ and C3H/HeN mice as shown by IgG levels to CGG equal to those of mice immunised with CGG alone. This harsh alkali treatment would probably detoxify LPS (Seid et al., 1981), which is consistent with the lack of adjuvant effect in the C3H/HeN mice, depolymerise nucleic acid, de-O-acyelate other lipid components and denature protein. The CPS itself is relatively stable, although it may be slightly modified, and any O-acetyl substituents or lipid-linked anchors are lost.

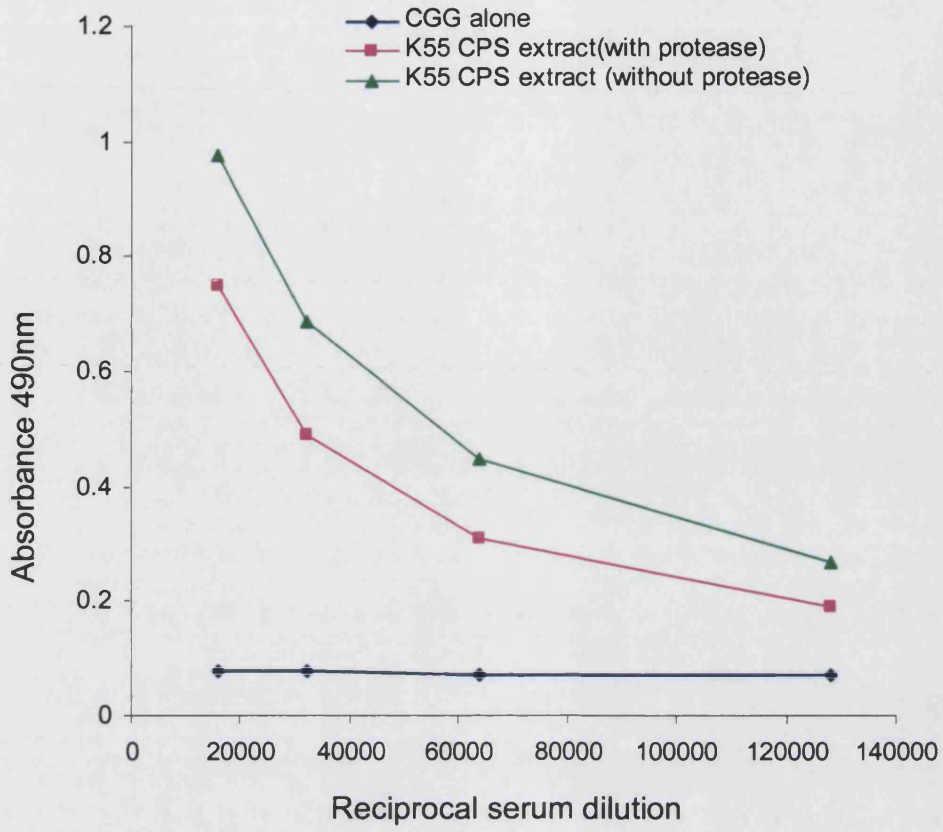


Figure 4.9 The effect of protease treatment on the adjuvanticity of K55 CPS extract in the C3H/HeJ mice

CPS was digested with Subtilisin for 18h at 37°C as described in section 2.2.1c. Groups of three mice were injected by the intra peritoneal route with either 100μg of CGG with no adjuvant, or together with untreated or protease treated K55 CPS. Mice were bled after 21 days. The serum was pooled and IgG to CGG was measured by ELISA as described in Section 2.2.4g. The data shown are means of duplicate wells and are from one representative study out of two.

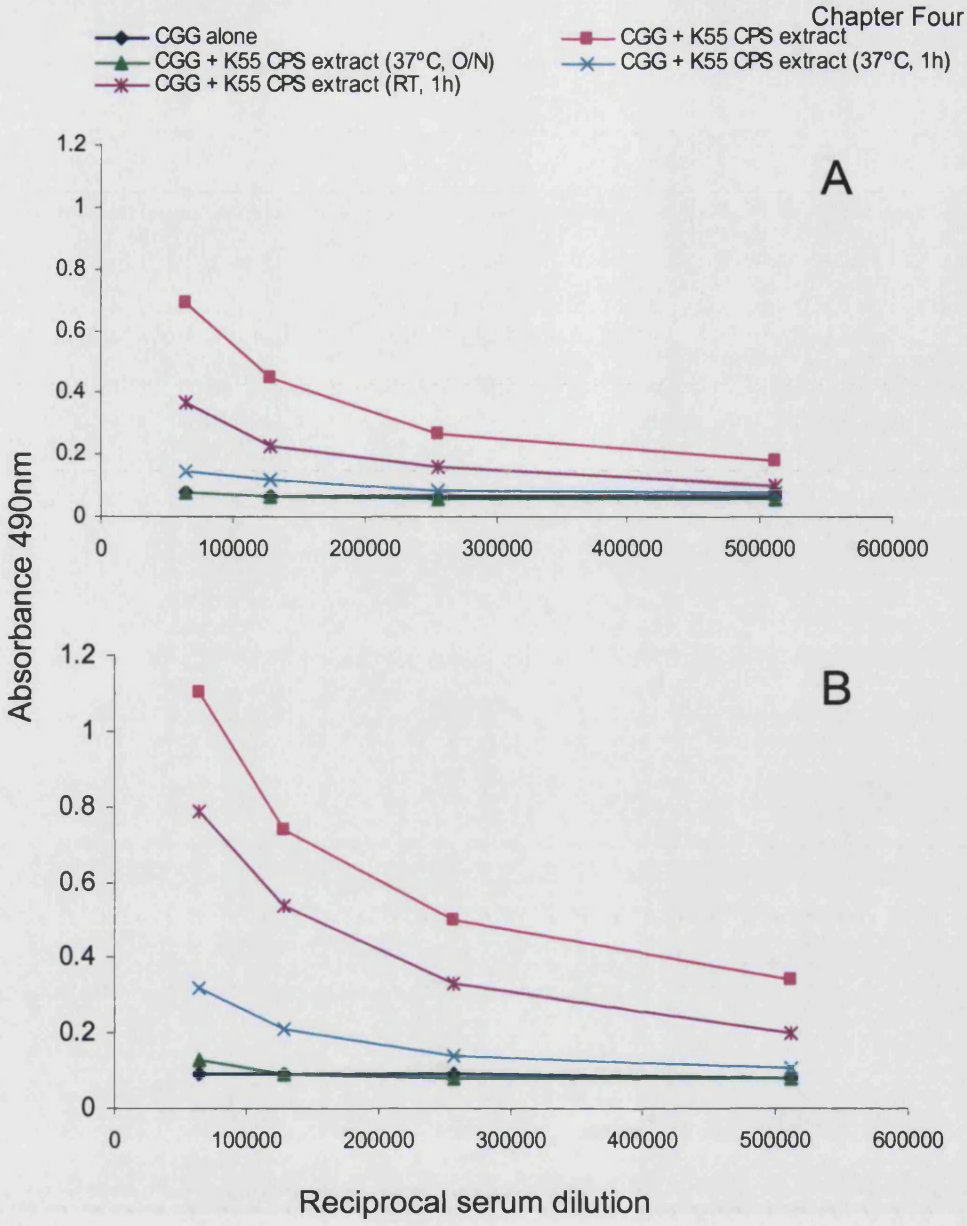


Figure 4.10 Effect of different alkali treatments on the adjuvanticity of the CPS extract from *K. pneumoniae* K55.

K55 CPS extract (150μg) was suspended in 750μl 0.1M NaOH and then incubated for either 1h at room temperature, 1h at 37°C, or 18h at 37°C. Groups of three C3H/HeJ (A) and C3H/Hen (B) mice were immunised by the intra peritoneal route with 100μg of CGG mixed with 5μg K55 CPS extract either untreated or alkali treated. The mice were bled 21 days post immunisation. Serum was pooled and then analysed for IgG to CGG by ELISA, as described in Section 2.2.4g. These data shown are means of duplicate wells and are from one representative study out of two.

4.5 The humoral response to capsular polysaccharides from *Klebsiella pneumoniae*

In addition to assessing the adjuvanticity of *K. pneumoniae* CPS extracts, the serum antibody response to *K. pneumoniae* CPSs K17, K52 and K55 was also studied. This was to determine the nature of the response to the adjuvant. Mice were immunised with *K. pneumoniae* CPS pool 1 (very pure CPS). C3H/HeJ mice were used to control for LPS contamination because LPS has adjuvant properties and could have an effect on the antibody response to CPS. Investigating the antibody responses in both C3H/HeJ and wild type (C3H/HeN) mice provides insights into the effect of LPS on the antibody response to *Klebsiella* polysaccharides.

Figure 4.11 shows the serum IgM levels in C3H/HeN and C3H/HeJ mice immunised with *K. pneumoniae* K17 and K52 CPS pool 1. Both C3H/HeN and C3H/HeJ mice produced IgM in response to K17 and K52 CPS pool 1, although the response in the C3H/HeJ mice was lower. It was possible that this was due to the presence of LPS in the CPS pool 1 and the LPS had an adjuvant effect upon the anti-polysaccharide response. K55 gave a very poor IgM response in both C3H/HeN and C3H/HeJ mice (data not shown), but an IgG response was detected as shown in Figure 4.12A. Similar to the IgM response, IgG levels were greater in C3H/HeN mice compared to C3H/HeJ mice. This was the case for pool 1 of K55 and K52 CPS (Figure 4.12B), although no IgG was detected for K17 CPS pool 1.

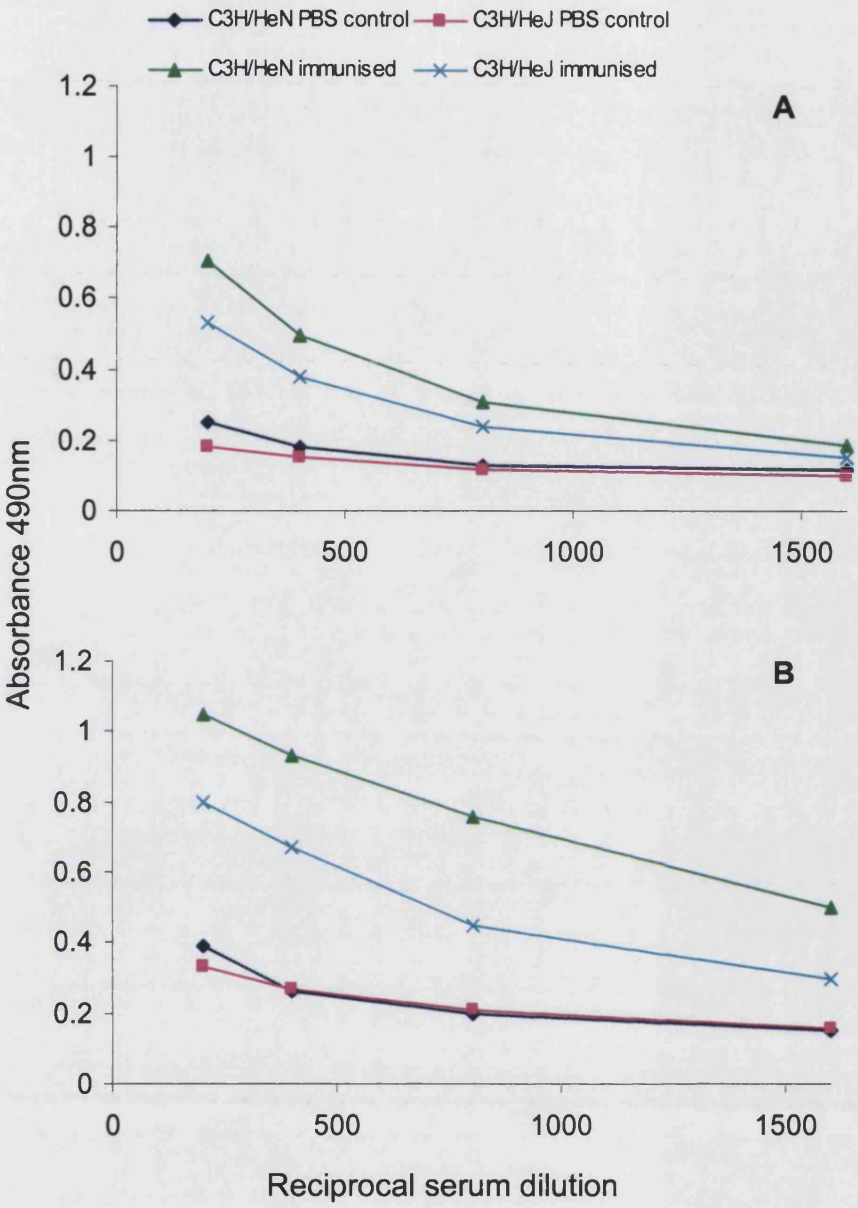


Figure 4.11 Serum IgM levels to *Klebsiella pneumoniae* capsular serotypes K17 and K52 in C3H/HeN and C3H/HeJ mice.

K. pneumoniae CPSs K17 and K52 were extracted and purified by gel filtration chromatography on a TSK G5000 column as described in the methods section. Groups of three C3H/HeN and C3H/HeJ mice were immunised with 5μg of K17 (A) and K52 (B), mice were bled 21 days post immunisation. Serum was pooled and assessed for IgM by ELISA as described in Section 2.2.4f. These data shown are means of duplicate wells and are from one representative study out of two.

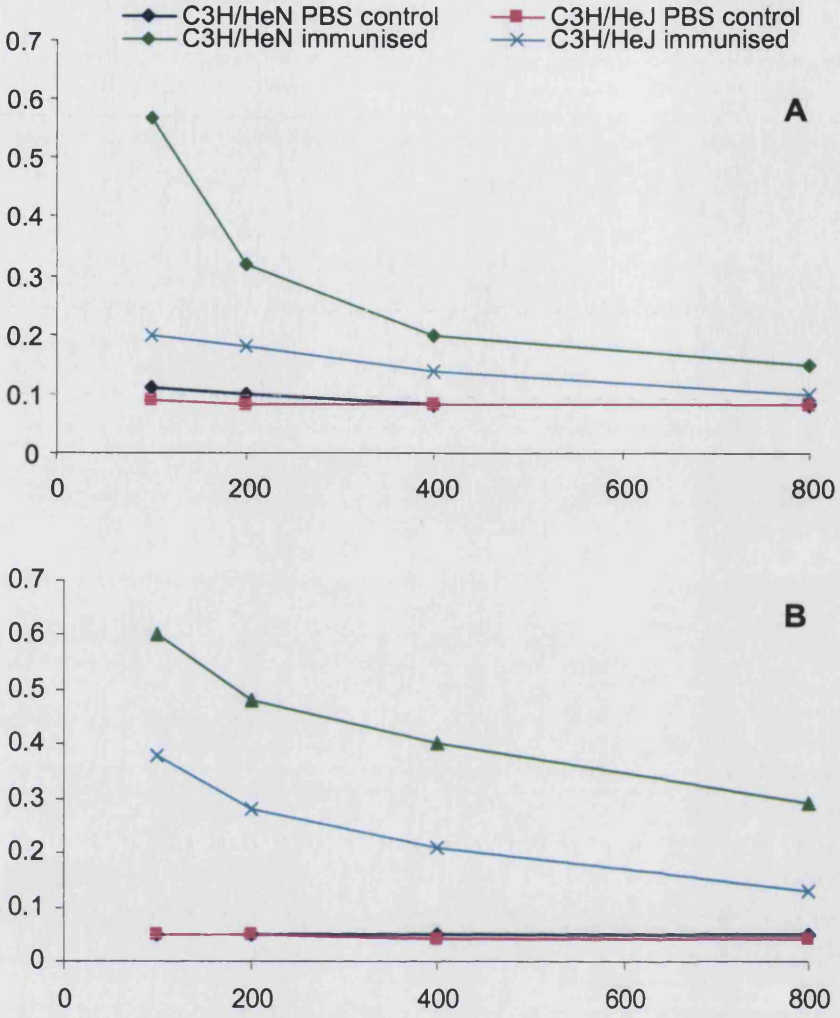


Figure 4.12 Serum IgG levels to *Klebsiella pneumoniae* capsular serotypes K55 and K52 in C3H/HeN and C3H/HeJ mice.

K. pneumoniae CPSs K55 and K52 were extracted and then purified by gel filtration chromatography as described in the methods section. Groups of three mice were immunised with 5μg of K55 (A) and K52 (B). Mice were bled 21 days after immunisation, serum was pooled and analysed for IgM by ELISA as described in Section 2.2.4f. These data shown are means of duplicate wells and are from one representative study out of two.

4.6 Discussion

From reports in the literature it is not clear whether *K. pneumoniae* CPSs alone have any immunostimulatory activity, or contribute in any way to the adjuvanticity of CPS extracts. It is possible that co-extracted components are contributing to adjuvant activity. Microbial products have major immunomodulatory effects on the immune system; such components include bacterial DNA containing unmethylated CpG motifs, LPS, outer membrane proteins (Omp), porins and lipoproteins. OmpA from *K. pneumoniae* has been shown to bind to and activate human macrophages (Soulas et al., 2000) as well as dendritic cells and then signal through TLR2 to induce maturation (Jeannin et al., 2000). Porin OmpK36 from *Klebsiella* can activate the classical complement pathway (Alberti et al., 1996). For LPS, bacterial lipoproteins and CpG motifs it is well recognised that these constituents have a major effect on the immune system via toll like receptors, TLR4, TLR2 and TLR9 respectively. Bacterial DNA (Klinman et al., 1999), lipopeptides (Sieling et al., 2003) and LPS (Skidmore et al., 1975; Schenck et al., 1969) possess adjuvant properties. However the possibility that *K. pneumoniae* CPSs have unique biological properties cannot be ruled out. This may be mediated, for example, through the presence of a lipid-linked anchor or through an unusual structural feature of the polysaccharide. The latter appears to be the case for β-glucan, which is immunostimulatory and recognised through Dectin-1 expressed on macrophages and dendritic cells (Brown and Gordon, 2001). Polysaccharides, when used as human vaccine antigens for example, are normally inert and do not induce an overt inflammatory reaction. If immunomodulatory effects can be demonstrated for certain polysaccharides,

these would potentially be safe when used as an adjuvant. For example, Biostim is well tolerated in humans but has desirable adjuvant properties (Profeta et al., 1987).

To confirm the adjuvanticity of *K. pneumoniae* CPS extracts, mice hyporesponsive to LPS (C3H/HeJ) were immunised with K55, K1 and K3 CPS extracts. All CPS extracts enhanced the IgG response to CGG independent of LPS, and were therefore deemed to have additional adjuvant properties (Figure 4.3). Although a response was seen independent of LPS, the IgG levels were greater in wild type mice. This suggests that LPS did contribute to adjuvanticity of CPS extracts. An increase in dose of CPS extract from 5 to 50μg did have an effect on the magnitude of the IgG response to CGG in C3H/HeJ mice, but 5ug of CPS still gave a good detectable response which suggests that adjuvant activity is present at low doses. Other studies in the literature have shown that *K. pneumoniae* CPS extracts enhance the antibody response to bovine serum albumin (BSA) (Nakashima, 1972; Nakashima and Kato; 1975, Nakashima et al., 1971), but none of these studies have shown this to be independent of LPS.

To determine the contribution of the CPS component in the *K. pneumoniae* CPS extracts, methods of purification of CPS were established as shown in Chapter 3. After purification the resulting *K. pneumoniae* CPS pool 1 was a very pure high MW CPS preparation. CPS pool 2 contained protein, LPS and lower MW carbohydrate from the CPS extract. The adjuvanticity of these preparations was established in C3H/HeJ mice. No IgG response to CGG

was observed in C3H/HeJ mice when *K. pneumoniae* CPS pool 1 from strains K55, K52 and K17 were used as adjuvants. An antibody response was observed when these same CPS pool 1 were used in C3H/HeN mice, which implies that the only component in the pure CPS preparation to have adjuvant activity was LPS. These results almost certainly confirm that high MW *K. pneumoniae* CPSs K55, K52 and K17 do not have the ability to enhance antibody responses to model protein antigens. The adjuvant activity appears to be due to *K. pneumoniae* CPS pool 2 which contained mostly LPS and protein. When either K55 or K52 CPS pool 2 was used as an adjuvant in C3H/HeJ mice, an increase in the IgG response to CGG was observed. Therefore, there is an undefined component (s) within pool 2 which can augment antibody responses. These observations are quite significant as certain studies in the literature almost certainly attributed these activities directly to the CPS.

To further establish the nature of the adjuvant, the effect of protease and alkali treatments were studied. Protease treatment of the *K. pneumoniae* K55 CPS extract resulted in a slight reduction in the antibody response to CGG, suggesting that a protein, lipoprotein or glycoprotein contributed to adjuvanticity. Increasing time and temperature of incubation of the CPS extract with NaOH also resulted in a reduction of adjuvant properties. Harsh alkali treatment would denature protein and DNA and detoxify LPS as shown by the lack of response in C3H/HeN mice. K55 CPS is fairly stable but could be modified by loss of its O-acetyl group and, if present, lipid anchors. These results similarly suggest that the CPS itself does not function as an adjuvant,

although it is possible that modifications to the CPS have altered its properties. Milder alkali treatment reduced the enhancement of the IgG response to CGG but did not completely abrogate it.

LPS from *K. pneumoniae* capsular serotypes K1 (01), K3 (02) and K55 (03) were studied. The LPS O antigens of this species display very limited structural variation with only 9 recognised O serotypes. Three main serotypes consist of linear polymers of D-galacto-*pyranose* residues (galactan II) or both D-galacto-*pyranose* and D-galacto-*furanose* (galactan I) (serotypes 01 and 02) and linear polymers of α-linked D-Man residues (serotype 03). Serotype 03 appears to be the most interesting. When K55 LPS was tested as an adjuvant in C3H/HeJ mice the IgG response to CGG was enhanced, but when K1 or K3 LPS was used the antibody response to CGG was absent. LPS with a mannose-rich O-polysaccharide is 100 times more potent at activating the complement system than LPS containing other O-antigen structures (Kido et al., 1985). It has also been shown to possess greater adjuvant activity by enhancing antibody responses to protein antigens such as bovine gamma globulin (Kido et al., 1985) and ovalbumin (Ohta et al., 1985). The latter study was carried out with LPS hyporesponsive mice. This observation creates a possible problem for assessing the adjuvant properties of *K. pneumoniae* CPS extracts from K55:03 in C3H/HeJ. An IgG response to CGG could be observed in C3H/HeJ mice when K55:03 LPS was used an adjuvant, so it was difficult to determine whether the adjuvant properties of K55 CPS extract in C3H/HeJ mice are due to LPS or not. However it is important to note that mice were immunised with 5μg of LPS and 5μg of CPS

containing only 600ng of LPS, and both gave similar IgG responses to CGG (see Figure 4.3). In retrospect it would have been valuable to look at the dose response of K55 LPS, to see the magnitude of antibody response to CGG.

The humoral response to *K. pneumoniae* CPS pool 1, pure CPS preparation, showed variation among capsular serotypes. An IgM response was observed for K17 and K52, but none for K55 in C3H/HeJ mice. K55 and K52 both produced IgG responses in C3H/HeJ mice. A study by Cryz and colleagues showed that the immunogenicity in mice varied amongst capsular serotypes, but all evoked IgG responses, but K17 CPS was not included in this study (Cryz et al., 1985). Although responses were observed in C3H/HeJ mice they were reduced compared to C3H/HeN mice. It is possible that LPS in the *K. pneumoniae* CPS pool 1 is having an adjuvant effect on the humoral response to the CPS.

In conclusion, it is unlikely that highly purified capsular polysaccharides from *K. pneumoniae* have adjuvant properties which result in augmentation of the humoral response to a soluble protein antigen. Components within CPS extracts other than LPS appear to have the ability to enhance antibody responses at very low doses, so are therefore very potent. The next stage is therefore to identify the component which has these immunostimulating properties and to determine whether this is a known or novel adjuvant.

Chapter Five

Results

Chapter Five

Possible mechanisms of adjuvant action of capsular polysaccharide extracts from *Klebsiella pneumoniae.*

5.1 Introduction

An adjuvant can be defined as any product (or combinations of components) which modulates the humoral or cellular immune responses against an antigen or pathogen. Vaccines come in several forms: live attenuated, replicating pathogens and non-replicating inactivated pathogens and their subunits. Subunit vaccines are considered to be the safest, but because of their poor or absent immunogenicity, they often require the addition of an adjuvant to induce an effective immune response. Currently only Alum is licensed for use in humans. Many microbial components are strong adjuvants, but are also associated with toxicity. Complete Freunds adjuvant, based on paraffin oil containing heat killed whole mycobacterial cells is a highly effective adjuvant, but is too toxic for human use (Billiau and Matthys, 2001). LPS is also an adjuvant but again associated toxicity prohibits its delibrate use in human vaccine formulations.

Although vaccine adjuvants are recognised as a group of immunomodulating agents, the mechanisms underlying their activities are generally poorly defined. There are several concepts that attempt to explain their mode of action. For example, certain adjuvants are able to convey long term presentation of the antigen (depot effect); others help to target immune cells

(e.g. by delivering antigens of a particulate nature to antigen presenting cells). Some adjuvants exhibit a capacity to elicit the production of different patterns of cytokines (i.e. Th1 or Th2) and/or enhance the levels of costimulatory molecules on APCs (Moingeon et al., 2001). Emulsion-based Alum and insoluble adjuvants are thought to work by helping to form a depot of antigen, although it has been suggested that Alum may be contaminated with LPS. Antigens and adjuvants are sequestered at the injection site and are released over a period of time to stimulate APCs such as macrophages and DCs. Particulate adjuvants, such as liposomes and immunostimulating complexes (ISCOMs), bind to antigens and form aggregates, which can then target APCs. More effective targeting of antigen can be achieved by using adjuvants with ligands that are recognised by receptors on APCs. For example the mannose receptor binds compounds that contain D-Man, GlcNAc or D-Fuc residues, such as those found on some saponin adjuvants (Cox et al., 1998). Therefore adjuvant-antigen complexes can be targeted to APCs via specific pattern recognition receptors.

Bacterial components such as LPS, lipoprotein, bacterial DNA, peptidoglycan and flagellin are recognised by TLRs. Pathogen recognition by TLRs results in rapid activation of innate immunity by inducing the production of proinflammatory cytokines and upregulation of costimulatory molecules. Activation of innate immunity subsequently leads to effective adaptive immunity. TLRs provide a critical link between innate and adaptive immunity and therefore have been considered as adjuvant receptors. Bacterial DNA contains immunostimulatory motifs that trigger an innate immune response

characterised by a predominantly Th1-type cytokines. These motifs consist of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines and are recognised by TLR 9. CpG DNA has been shown to augment the immune response to ovalbumin (Klinman et al., 1999) and hepatitis B surface antigen (McCluskie and Davis, 1999) in mice and rats respectively. It is for this reason that development of adjuvants has focussed on molecules that stimulate innate immunity through TLRs.

K. pneumoniae CPS extracts have been shown to have immunostimulatory properties, including the enhancement of antibody responses to normally non-immunogenic proteins (Nakashima et al., 1971; Nakashima and Kato, 1975; Nakashima, 1972). The mechanism of the immunostimulatory action of the CPS extracts is poorly understood. However, one such extract referred to as Biostim is used orally in humans to treat persistent bacterial bronchial infections. *In vitro* studies using mouse (Wood and Moller, 1984) and human (Martinez-Maza et al., 1985) cells have shown Biostim to be a strong polyclonal B cell activator. It also has the ability to activate human monocyte-derived macrophages directly and inhibit the intracellular multiplication of *Legionella pneumophilia* (Rajagopalan et al., 1987). The component(s) present in the polysaccharide extracts that possesses these activities have not been identified.

5.2 Objectives

The aim of the work described in this chapter was to investigate possible mechanisms by which *K. pneumoniae* CPS extracts enhance the antibody response to CGG.

5.3 Results

The results described in the following sections include different *K. pneumoniae* samples. A summary of preparations is shown in Table 5.1. In the case of CPS pool 2 which contained components co-extracted with CPS, a CPS concentration cannot be accurately determined; therefore an equivalent volume to that used for CPS pool 1 was used.

Table 5.1 *K. pneumoniae* preparations

K. pneumoniae	**Methods**	**Components**
CPS extract	CPS extracted as described in Section 2.2.1, subjected to ultracentrifugation and protease and nuclease digestion	CPS (typical % content) 10-15% protein 5% nucleic acid 1-2% LPS
CPS pool 1	CPS extracts separated on a TSK G5000 column, high MW pool.	Mostly CPS (very pure) 1% protein <0.10% nucleic acid 0.01% LPS
CPS pool 2	CPS extracts separated on a TSK G5000 column, lower MW pool.	Contains mostly protein and LPS from CPS extract.
LPS	Extracted and purified as described in the Section 2.2.2.	Very Pure

5.3.1 Proliferation of C3H/HeJ and C3H/HeN splenocytes in response to *K. pneumoniae* capsular polysaccharide extracts.

Studies were carried out to investigate the induction of proliferation of splenocytes by *K. pneumoniae* CPS extracts. These experiments were carried out using the spleens from C3H/HeN and C3H/HeJ mice. The use of

C3H/HeJ mice enabled us to study the effects of CPS extracts that are not due to LPS. Figure 5.1 shows the proliferation of C3H/HeJ and C3H/HeN splenocytes after stimulation for 72h with different concentrations of *K. pneumoniae* K55:O3 LPS. Incorporation of tritiated thymidine into DNA of dividing cells was used to indicate proliferation of splenocytes. C3H/HeN splenocytes proliferated in response to LPS in a dose dependent manner. In contrast, the C3H/HeJ splenocytes did not proliferate in response to K55 LPS. No significant difference was seen between the media only control (0µg/ml LPS) and stimulation with 0.1 - 10µg/ml LPS. These results confirm that the C3H/HeJ splenocytes do not proliferate in response to *Klebsiella* LPS.

Two *K. pneumoniae* capsular serotypes, K55 and K52 were investigated. In Chapter 4 it was shown that purified high MW CPSs from K55 and K52 (i.e. pool 1) did not have the ability to enhance an antibody response to CGG, but other components within the CPS extracts (i.e. pool 2) were responsible for this adjuvant effect. Two possible components contributing to adjuvanticity are LPS and DNA. Table 5.2 shows the concentration of these components present in K55 and K52 CPS extracts. Purified DNA and LPS from these two capsular serotypes were used separately to stimulate splenocytes in order to determine the level of proliferation induced by each component at concentrations similar to or greater than those found in CPS extracts.

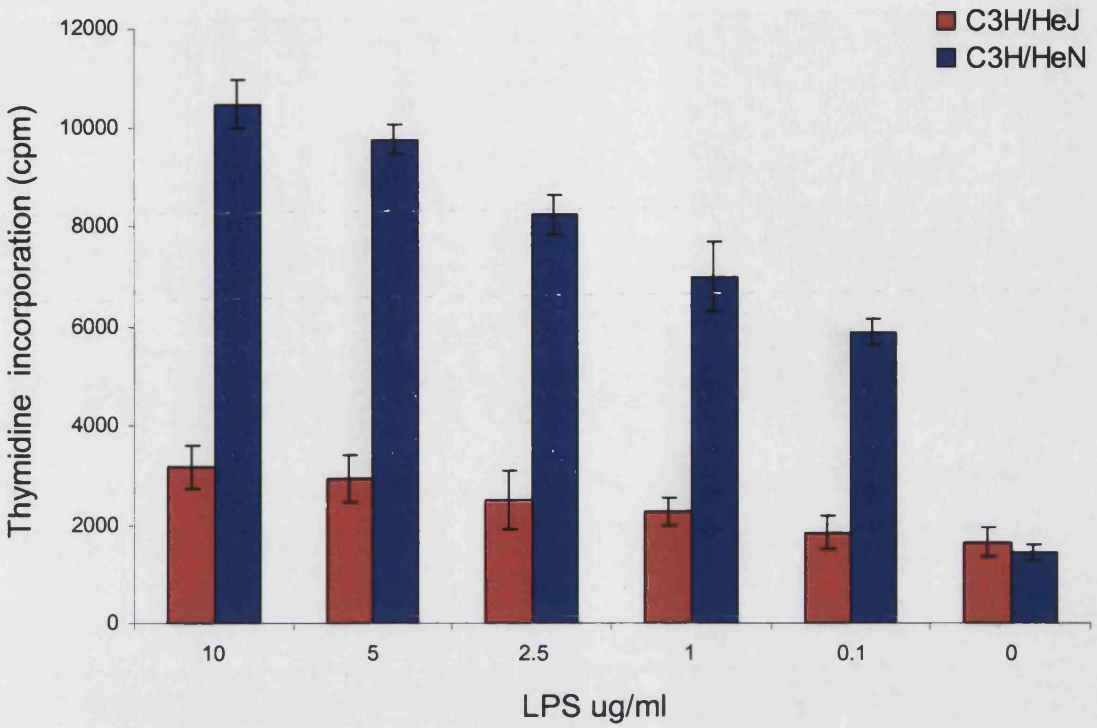


Figure 5.1 Proliferation of splenocytes from C3H/HeN and C3H/HeJ mice stimulated with LPS from *Klebsiella pneumoniae* serotype K55.

Spleens were taken from naïve C3H/HeN and C3H/HeJ mice and cell suspensions were prepared. Cells (2 x 10^5 /well) were cultured with medium alone or K55 LPS (0.1-10μg/ml). *K. pneumoniae* LPS was extracted by aqueous 45% (w/v) phenol and purified as described in the methods section. Cells were pulsed with tritiated thymidine after 72h of culture and were then frozen 18h later. Upon thawing proliferation was determined using a scintillation counter. Data represents the mean of three experiments ± s.e.m.

Table 5.2 Concentration of CPS, DNA and LPS in *K. pneumoniae* K55 and K52 CPS extracts.

Component	K55 CPS extract	K52 CPS extract
CPS	25µg/ml	25µg/ml
LPS	600ng/ml	50ng/ml
DNA	< 50ng/ml	<50ng/ml

The incorporation of tritiated thymidine into C3H/HeJ splenocytes after stimulation with K55 (A) and K52 (B) CPS extracts is shown in Figure 5.2. Both *K. pneumoniae* K55 and K52 CPS extracts induced the proliferation of C3H/HeJ splenocytes which was significantly different ($p<0.005$) from splenocytes stimulated with media only. Purified *Klebsiella* DNA and LPS failed to stimulate splenocyte proliferation, even at concentrations greater than those present in the extracts. This indicates that the LPS and DNA in the CPS extracts were not inducing the proliferation of C3H/HeJ splenocytes.

In order to investigate further the nature of the stimulatory components, *K. pneumoniae* K55 and K52 CPS extracts were subjected to a variety of procedures including further purification, protease and alkali treatments (Figure 5.3 and 5.4).

PMA and ionomycin were used as a positive control to show splenocytes from both C3H/HeJ and C3H/HeN had the ability to proliferate. This is illustrated in Figure 5.3A where both C3H/HeJ and C3H/HeN splenocytes proliferated to similar extents in response to PMA and ionomycin treatment.

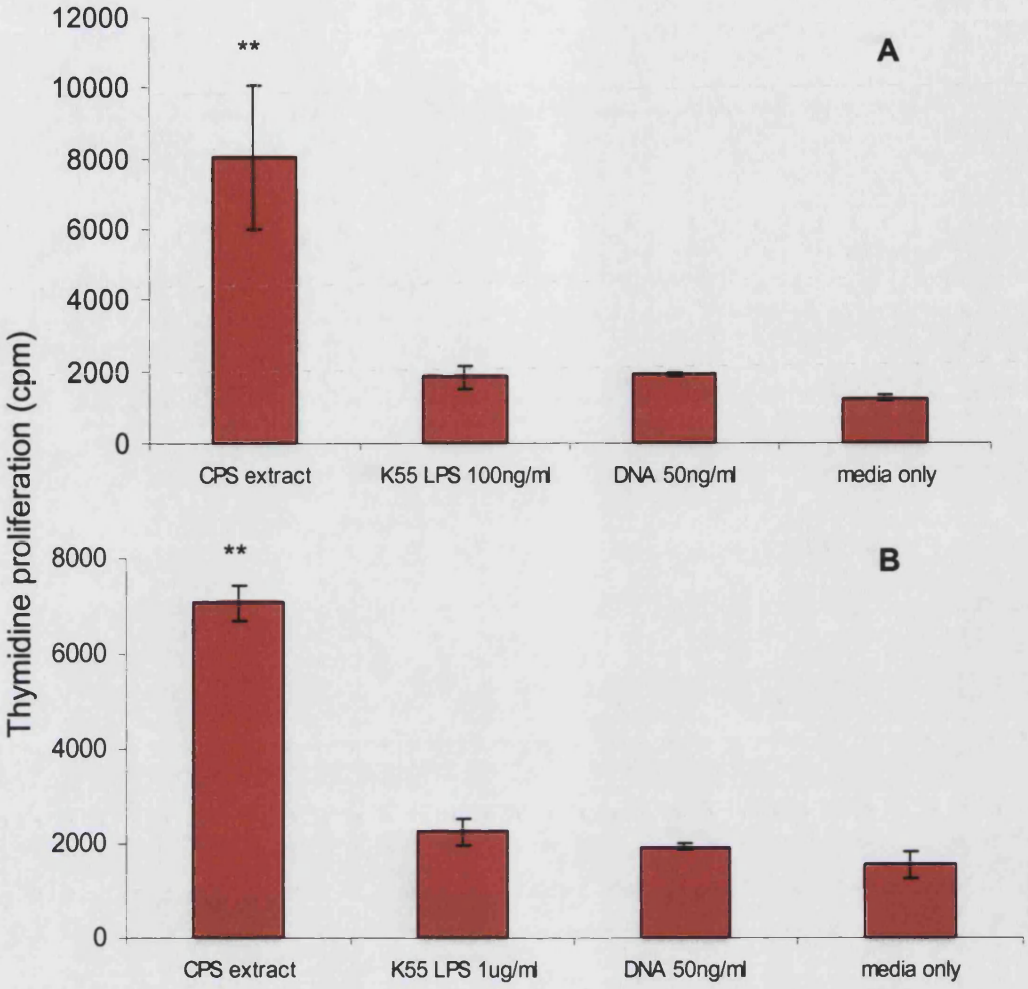


Figure 5.2 Proliferation of C3H/HeJ splenocytes stimulated with K52 and K55 CPS extracts.

Spleens were taken from naïve C3H/HeJ mice and a cell suspension was prepared. Cells were cultured with either media only, 25μg/ml K52 (A) or K55 (B) CPS extract, K55 LPS (100ng/ml – 1μg/ml) or K52 DNA (50ng/ml). DNA was prepared using a Qiagen DNA purification columns (Qiagen, West Sussex, U.K.) that bind DNA to an anion-exchanger resin under appropriate low-salt and pH conditions and elute DNA in high-salt conditions. Cells were pulsed with tritiated thymidine after 72h of culture and were frozen 18h later. Upon thawing proliferation was determined using a scintillation counter. Data represents the mean of 3 experiments ± s.e.m. (Media only $\neq$ sample;**, $p<0.005$).

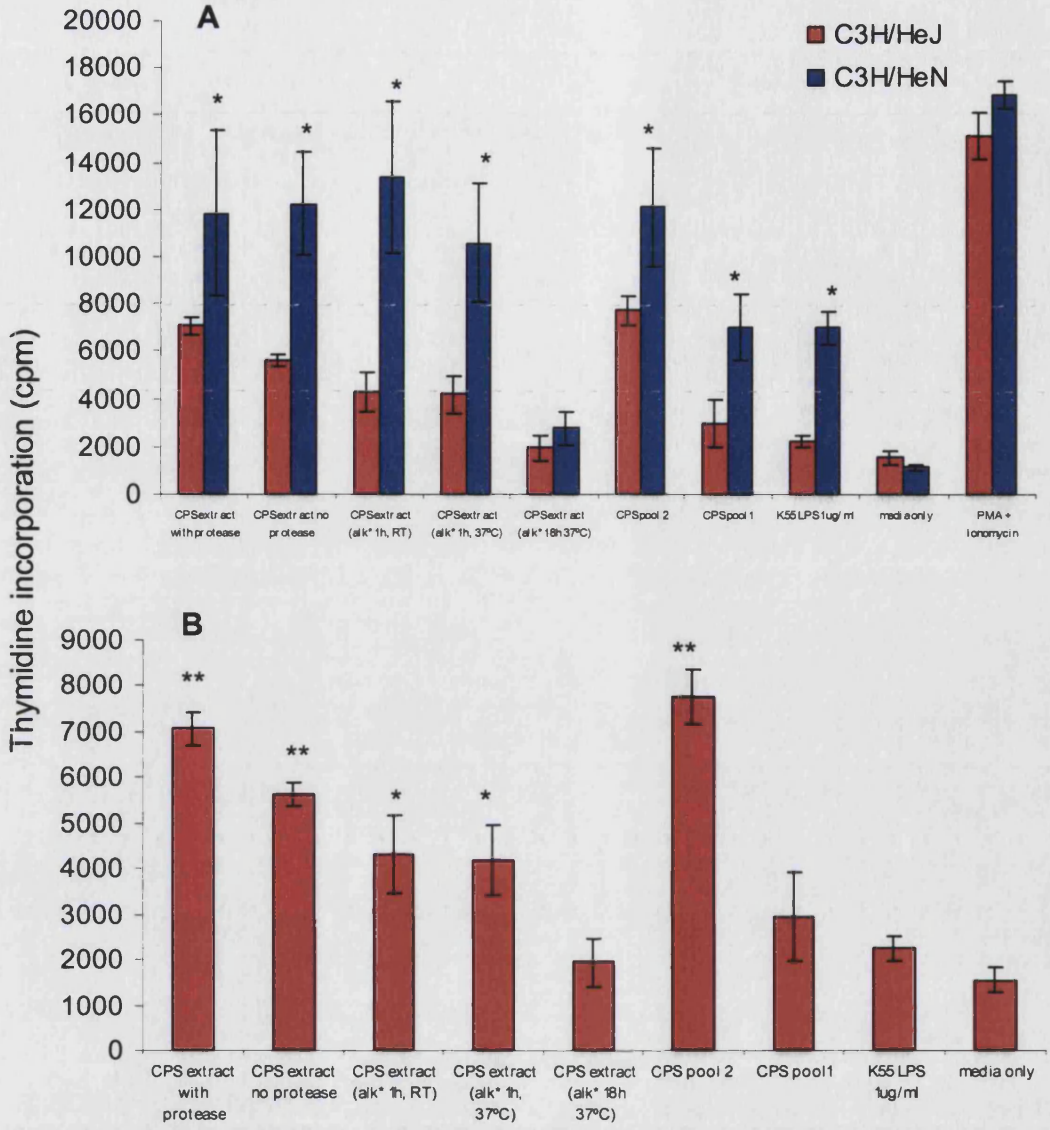


Figure 5.3 Proliferation of C3H/HeN and C3H/HeJ splenocytes stimulated with K55 CPS.

Spleens were removed from naïve C3H/HeN and C3H/HeJ mice and single cell suspensions were prepared. Cells (2 x 10^5 /well) were cultured with either medium alone, 1μg/ml K55 LPS, K55 CPS extract with or without protease treatment, CPS extract separated by gel filtration chromatography (CPS pool1 and CPS pool 2), or CPS extract subjected to alkali treatment. Alkali treatment of K55 CPS extract (150μg) was carried out in 0.1M NaOH (750μl) and incubated for either 1h at room temperature (alk* 1h RT), 1h at 37°C (alk* 1h 37°C) or 18h at 37°C (alk* 18h 37°C). All CPS samples were at 25μg/ml. Cells were pulsed with tritiated thymidine after 72h of culture and were frozen 18h later. Upon thawing proliferation was determined using a scintillation counter. Data represents the mean of 3 experiments ± s.e.m. For (A) C3H/HeN ≠ C3H/HeJ and (B) media only ≠sample; *, $p<0.05$, **, $p<0.005$.

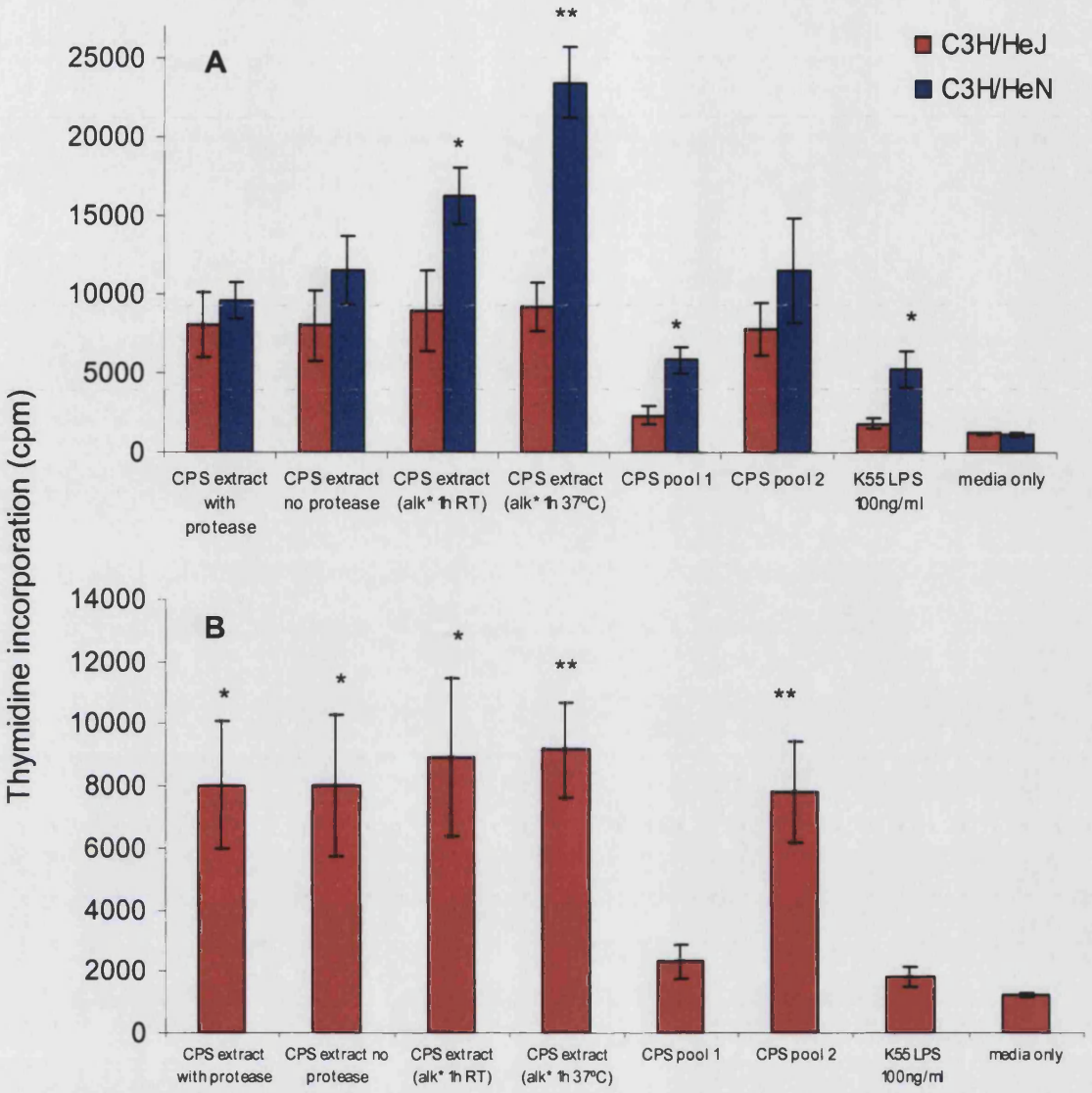


Figure 5.4 Proliferation of C3H/HeN and C3H/HeJ splenocytes stimulated with K52 CPS.

Spleens were removed from naïve C3H/HeN and C3H/HeJ mice and cell suspensions were prepared. 2 x 10^5 cells per well were cultured with K52 CPS extract, CPS extract subject to protease digestion, CPS extract separated on a TSK G5000 column (pool 1 and 2), and CPS extracts subject to alkali treatment. Alkali treatment of K52 CPS extract (150μg) was carried out in 0.1M NaOH (750μl) and incubated for either 1h at room temperature (alk* 1h RT) or 1h at 37°C (alk* 1h 37°C). Cells were also cultured with K55 LPS (100ng/ml) and with media alone. All CPS were at 25μg/ml. Assays were pulsed with tritiated thymidine after 72 hours of culture and were frozen 18h later. Upon thawing proliferation was determined using a scintillation counter. Data represents the mean of three experiments ± s.e.m. For (A) C3H/HeN ≠ C3H/HeJ and (B) media only ≠sample; *, $p<0.05$, **, $p<0.005$.

In response to treated or untreated K55 CPS, the proliferation of C3H/HeJ splenocytes was significantly ($p<0.05$) less than C3H/HeN splenocytes for all except the CPS extract treated with 0.1M NaOH for 18h at 37°C, where activity was similar to those stimulated with media only. These results suggest that all the K55 samples, except the one treated extensively with alkali, contained biologically active LPS that contributed to the proliferation of C3H/HeN splenocytes. The proliferation of splenocytes from C3H/HeJ mice, which is independent of LPS, is shown in Figure 5.3B. There was no significant difference in the level of proliferation between C3H/HeJ splenocytes stimulated with media only and those stimulated with either purified K55 LPS, *K. pneumoniae* K55 CPS pool 1 (very pure CPS) or K55 CPS extract subjected to extensive alkali treatment. Both K55 LPS and K55 CPS pool 1 induced proliferation in C3H/HeN splenocytes due to the presence of LPS. In contrast, *K. pneumoniae* K55 CPS pool 2 induced proliferation in C3H/HeJ mice ($p<0.005$). The active components present in CPS extracts can therefore be separated from and are independent of high MW CPS. After mild alkali treatment (0.1M NaOH for 1h at either RT or 37°C) the K55 CPS extract induced less proliferation than untreated K55 CPS extract. Interestingly, the proliferation of C3H/HeJ splenocytes is significantly greater ($p=0.01$) when K55 CPS extract was protease treated. This suggests that intact proteins are not responsible for the activity. It is possible that the increased proliferation is due to the production of active peptides, lipopeptides or glycopeptides following protease treatment.

Similar experiments were carried out with *K. pneumoniae* CPS K52 extract (Figure 5.4). Similar to results with K55 CPS extract, proliferation did not occur when CPS pool 1 and LPS were used to stimulate C3H/HeJ splenocytes but proliferation was observed in C3H/HeN splenocytes suggesting the level of LPS contained in the pool 1 was still sufficient for activity. Consistent with results for K55, protease digestion of the K52 CPS extract had little effect on the proliferation of C3H/HeN splenocytes. However, unlike K55 CPS extract, protease treatment of K52 CPS extract did not increase proliferation in C3H/HeJ splenocytes. Surprisingly, mild alkali treatment of K52 CPS extract resulted in an enhanced proliferation of C3H/HeN splenocytes compared to untreated K52 CPS extract. The reason for this is not clear. It was observed that alkali treatment resulted in reduced viscosity of the CPS extracts which may allow active components greater accessibility to the responsive immune cells.

In summary, both K52 and K55 CPS extracts can induce proliferation of mouse splenocytes independent of LPS, but this activity is not due to very high MW CPS in pool 1.

5.3.2 Proliferation of C3H/HeJ and C3H/HeN B and T cells in response to *K. pneumoniae* capsular polysaccharide extracts.

It has been shown that C3H/HeJ splenocytes proliferate in response to both *K. pneumoniae* K52 and K55 CPS extracts. To determine which cell type proliferates, splenocytes were labelled with CFSE. After labelling, splenocytes were cultured with various stimuli for 72h and then labelled with either anti-B220 or anti-CD3 antibodies, markers of B and T cells

respectively. When cells proliferate, the overall intensity of CFSE labelling is reduced with each round of cell division. This is shown as an individual peak of reduced intensity. Figure 5.5 shows the lack of proliferation of T cells in response to K52 and K55 CPS extracts. C3H/HeN and C3H/HeJ T cells did not proliferate in response to either K55 or K52 CPS extracts with their profiles being identical to that of splenocytes stimulated with media only. Similar results were observed when splenocytes were stimulated with DNA and LPS from *K. pneumoniae*. As with the splenocyte proliferation assays using thymidine incorporation, the concentrations of LPS and DNA used were greater than those present in CPS extracts. PMA and ionomycin were used together as a positive control due to its ability to induce the proliferation of T cells. The results show that *K. pneumoniae* CPS extracts have no effect on T cell proliferation.

Figure 5.6 shows the proliferation of B cells in response to CPS extracts. The B cells in the C3H/HeN splenocyte population proliferated in response to K55 and K52 CPS extracts, and LPS and DNA from *K. pneumoniae*. B cells from C3H/HeJ mice also proliferated in response to both K52 and K55 CPS extracts, although proliferation was greater for K52 CPS extract. No proliferation of B cells was observed when stimulated with *Klebsiella* DNA at 50ng/ml but some limited proliferation could be observed with 1µg/ml DNA. The DNA preparation was found to contain LPS which could account for the good proliferation of B cells seen with C3H/HeN splenocytes. As both K55 and K52 CPS extracts used in this experiment contained less than 1µg/ml LPS and 50ng/ml DNA (Table 5.2), these results suggest that the

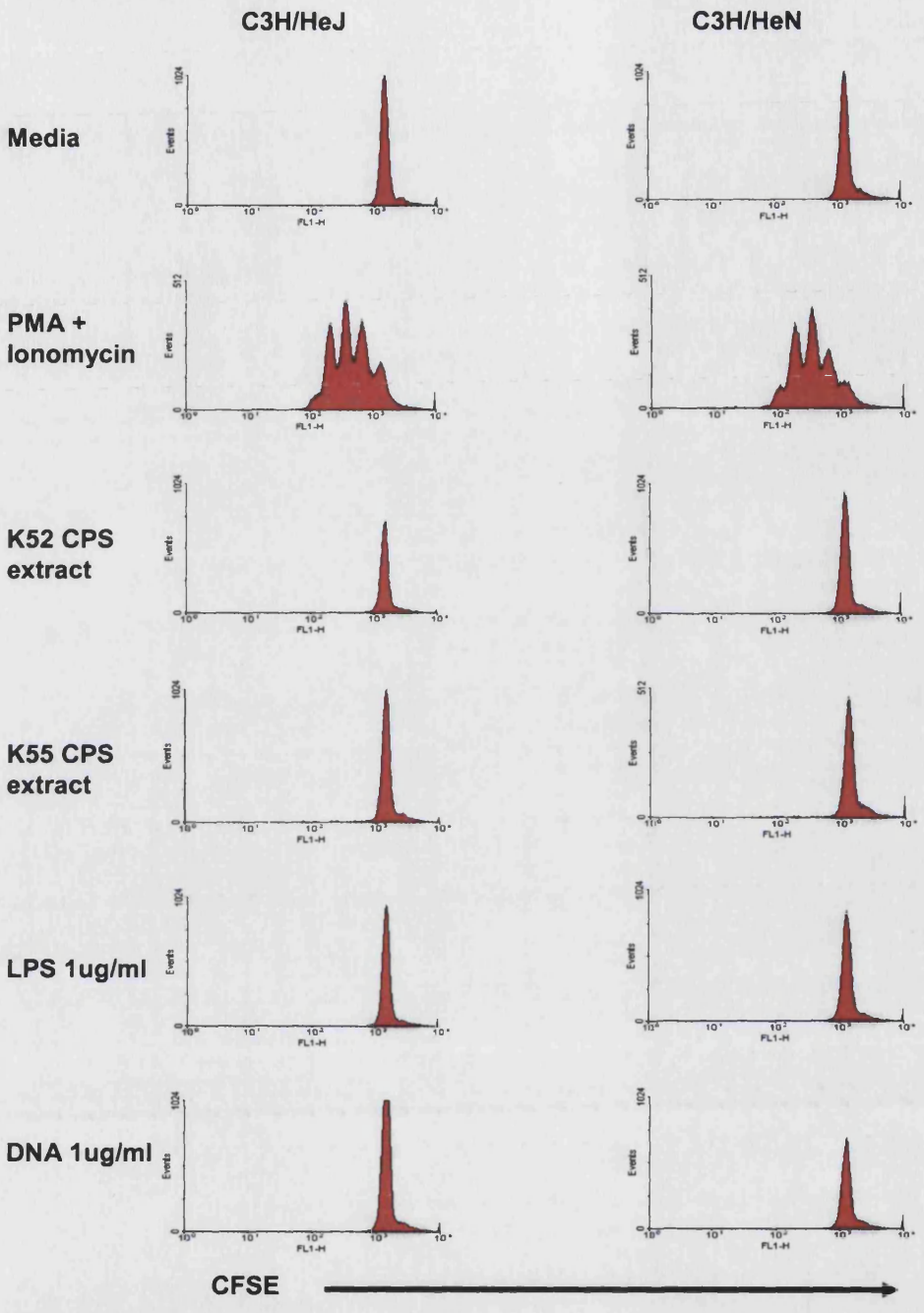


Figure 5.5 CFSE analysis of T cell proliferation in response to K52 and K55 CPS extracts.

Spleens from naive C3H/HeN and C3H/HeJ mice were taken and cell suspensions were prepared. Cells (1x 10^7 cells/ml) were labelled with 5μM CFSE at 37°C for 10 min. Cells were then cultured with either K52 DNA, K55 LPS, K55 or K52 CPS extracts (25μg/ml), PMA (5ng/ml) + Ionomycin (200ng/ml), or media alone. After 72h, cells were labelled with anti-CD3 antibody and examined by flow cytometry. Gates were placed around the CD3 positive population to determine proliferation of these cells. Cell divisions result in reduction of the CFSE fluorescence intensity. The experiment was repeated 4 times, and one representative data set is shown.

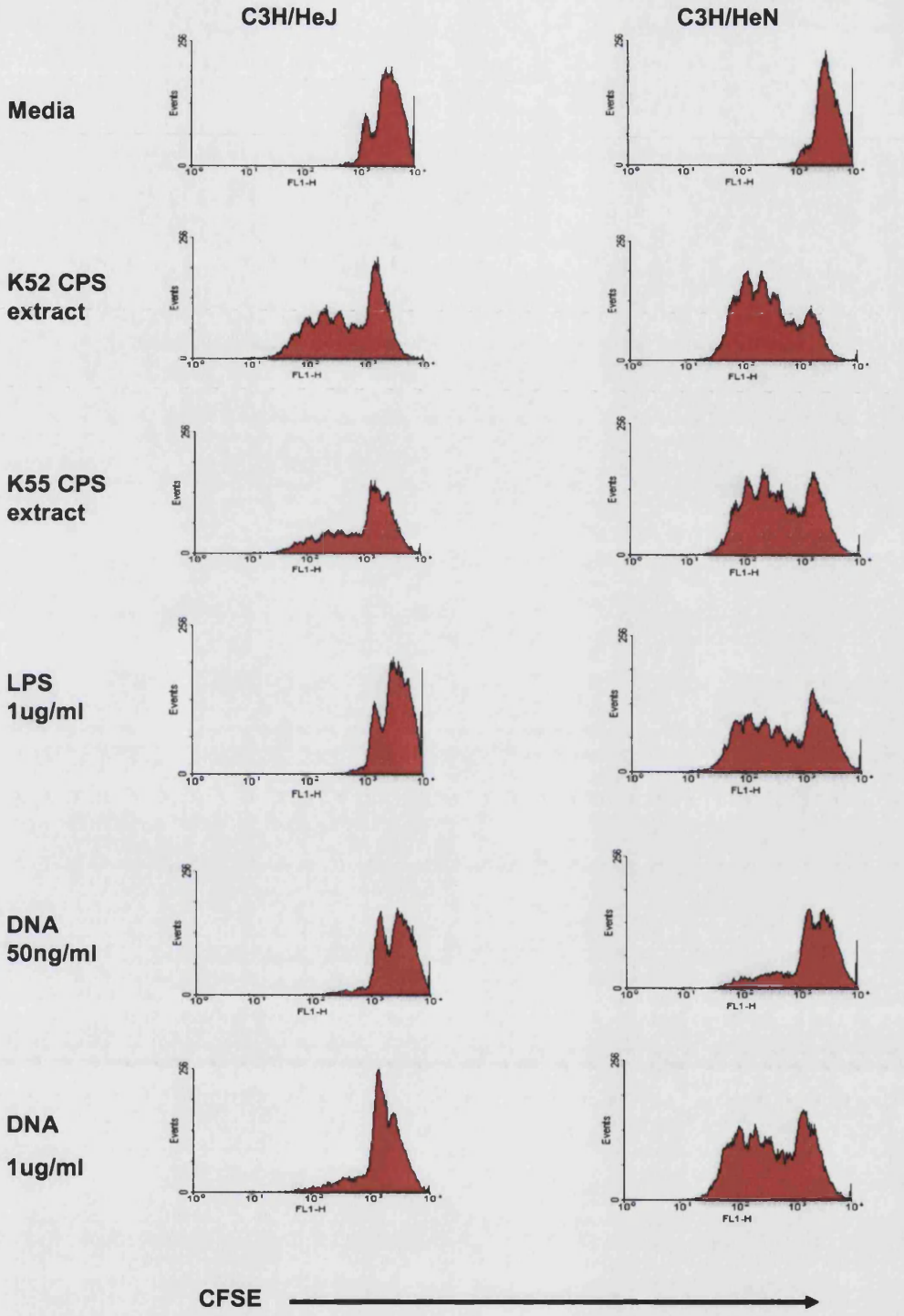


Figure 5.6 CFSE analysis of B cell proliferation in response to K52 and K55 CPS extract.

Spleens were taken from naïve C3H/HeJ and C3H/HeN mice and single cell suspension were prepared. Cells (1 x 10^7cells/ml) were labelled with 5μM CFSE at 37°C for 10 min. Cells were washed and then cultured for 72h with media alone, K52 DNA, K55 LPS, K55 or K52 CPS extracts (25μg/ml). Cells were then labelled with anti-B220 antibody and examined by flow cytometry. Gates were placed around the B220 positive population to determine proliferation of these cells. Cell divisions result in reduction of the CFSE fluorescence intensity. These data are from one representative study out of four.

proliferation of B cells was likely due to a component (s) in the CPS extracts which was not LPS or bacterial DNA.

5.3.3 Cytokine production from C3H/HeJ splenocytes after stimulation with *K. pneumoniae* capsular polysaccharide extracts.

The cytokine profiles induced by various stimuli were determined. Splenocytes from C3H/HeJ mice were cultured with a range of samples. After 48h supernatants were removed and analysed using a cytokine Cytometric Bead Array (CBA) assay. Figure 5.7 shows the release of cytokines from C3H/HeJ splenocytes when stimulated with K55 and K52 CPS extracts (panels A and B, respectively). K55 CPS extract induced the release of TNF-α, IFN-γ, MCP-1, IL-10 and IL-6. Very little cytokine release was observed from splenocytes stimulated with K55 LPS or with media alone. K52 DNA induced the release of various cytokines but these were of a significantly lower concentration than those induced with K55 CPS extract. Similar results were observed for the K52 CPS extract, with the release of TNF-α, IFN-γ, MCP-1, IL-10 and IL-6 by splenocytes. Minimal cytokine production was shown for splenocytes stimulated with K55 LPS, media only and K52 CPS pool 1. Most cytokine production was induced by K52 CPS pool 2. This suggests and consistent with other studies in this thesis that most of the immunostimulatory activity of CPS extracts is found in the CPS pool 2, rather than the highly purified CPS in pool 1. The immunomodulatory effects are unlikely due to LPS contamination as the studies were carried out in C3H/HeJ mice. The release of IL-2, IL-4, IL-5 and IL-12p70 was also analysed, but none of these cytokines were detected in any of the supernatants. The release of IL-12p70 in particular was studied at various

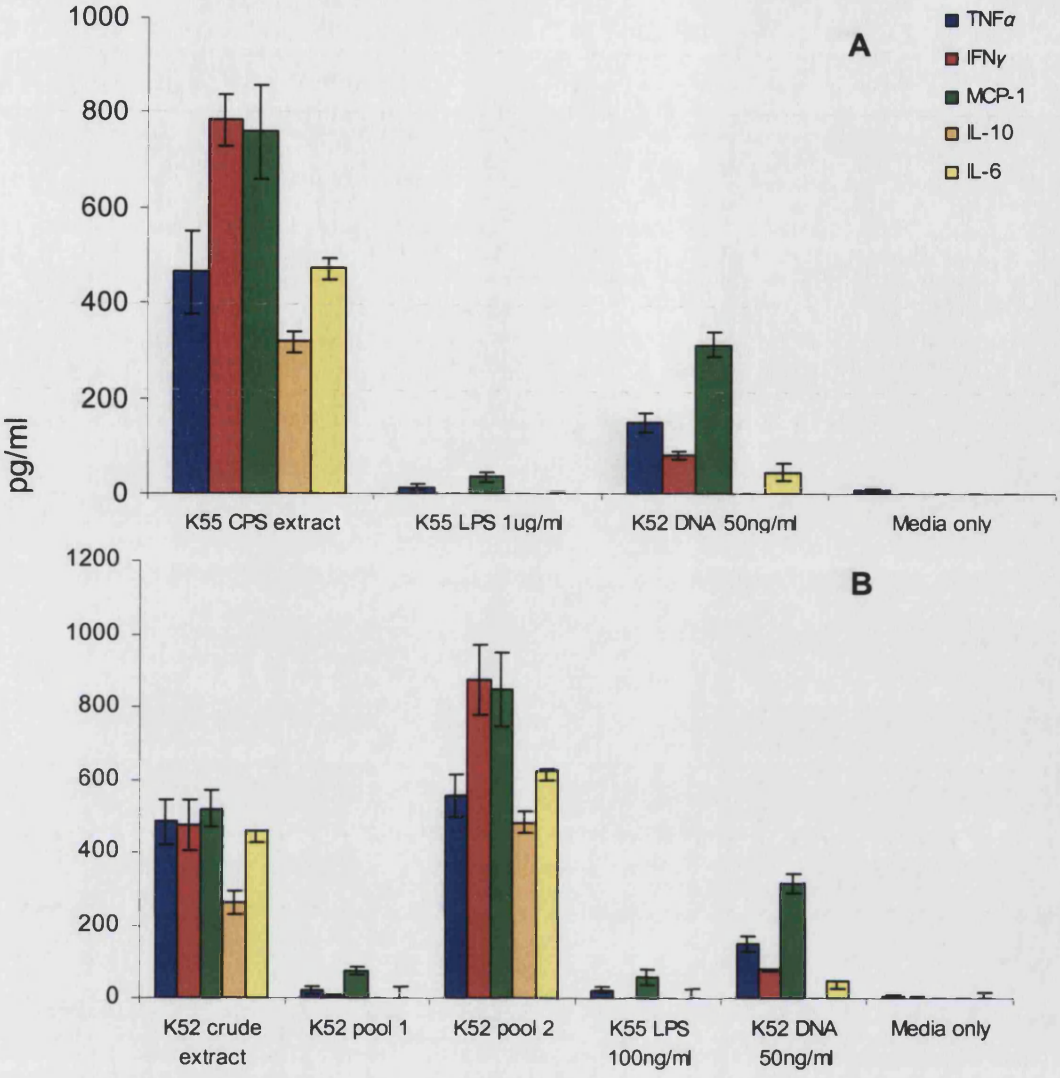


Figure 5.7 Cytokine release from C3H/HeJ mouse splenocytes after culture with K52 and K55 CPS extracts.

Spleens were removed from naïve C3H/HeJ mice and cell suspensions were prepared. Cells (2 x 10^5 /well) were cultured with 25μg/ml K55 (A) or K52 (B) CPS extracts or pooled samples generated after gel filtration chromatography. Cells were also cultured with LPS, DNA, or media alone. Supernatants were removed after 48h and then analysed for cytokines using a cytokine Cytometric Bead Array (CBA) assay (BD Biosciences, Pharmingen). Data represents the mean of three experiments ± s.e.m.

time points (18h, 2 days and 3 days). Both ELISA and CBA assays failed to detect the presence of IL-12p70 in any of the supernatants.

Preliminary studies were also carried out to determine cytokine release stimulated by known TLR2 agonists, MDP (N-acetylmuramyl-L-alanyl-D-isoglutamine) and Pam3Cys (Pam3Cys-Ser(lys)$_4$ HCl). Figure 5.8 shows the release of cytokines from C3H/HeJ splenocytes after stimulation with MDP and Pam3Cys. *K. pneumoniae* K52 and K55 CPS extracts were included for comparison. All stimuli were used at 25μg/ml. Very little cytokine production was observed for splenocytes cultured with either MDP or media alone. Pam3Cys induced the release of TNF-α, IFN-γ MCP-1, IL-6 and IL-10, but less IFN-γ was produced compared to both K52 and K55 CPS extracts.

5.4 Discussion

Chapter 4 in this thesis confirmed that *Klebsiella* CPS extracts augmented the antibody response to CGG, and provided strong evidence that the highly purified CPS in pool 1 is not the active component. This chapter examined the effect of these polysaccharide extracts on splenocyte populations. Both K52 and K55 CPS extracts induced the proliferation of splenocytes as shown by thymidine incorporation. Further studies using CFSE-labelled splenocytes confirmed that B cells, but not T cells proliferated in response to the *Klebsiella* CPS extracts. The component responsible for its effect on B cells has yet to be identified, but *Klebsiella* CPS extracts are known to contain both LPS and bacterial DNA, which have been reported to be polyclonal activators of B cells. Unmethylated CpG dinucleotides found in bacterial

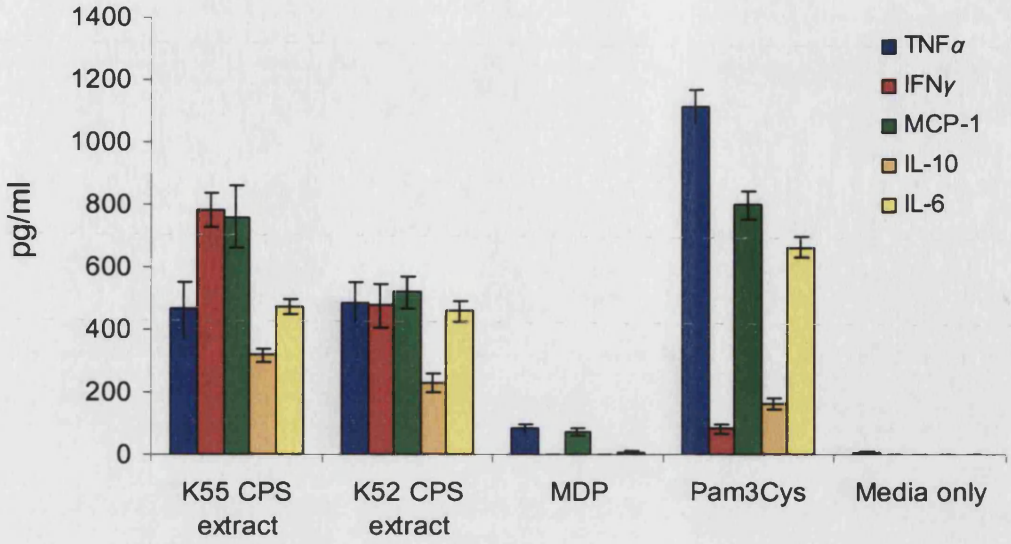


Figure 5.8 Cytokine release from C3H/HeJ mouse splenocytes after culture with K55 and K52 CPS extracts, Pam3Cys and MDP.

Spleens from naïve C3H/HeJ mice were removed and single cell suspensions were prepared. Cells (2 x 10^5/well) were cultured with 25μg/ml of K52 or K55 CPS extract, Pam3Cys (Pam3Cys-Ser(lys)$_4$HCL), or MDP (N-acetylmuramyl-L-alanyl-D-isoglutamine). Supernatant was removed from each well 48h later. Cytokines were analysed by a cytokine Cytometric Bead Array (CBA) assay (BD Biosciences Pharmingen). Data represents the mean of three experiments ± s.e.m.

DNA induce murine B cell proliferation and secretion of immunoglobulin *in vitro* and *in vivo* (Krieg, 2003, Messina et al., 1991). LPS can also induce the proliferation of B cells (Jaworski et al., 1982). In order to determine whether LPS in the CPS extracts was contributing to the proliferation of B cells, experiments were performed in LPS-hyporesponsive (C3H/HeJ) mice. *Klebsiella* LPS failed to induce proliferation of C3H/HeJ splenocytes. C3H/HeJ splenocytes cultured with *Klebsiella* DNA at an equal or greater concentration to that present in the CPS extracts, showed very limited proliferation compared with CPS extracts. This confirmed that DNA and LPS present in the CPS extracts were not responsible for the proliferation of B cells.

Klebsiella LPS used in these studies was type 03. This LPS contains a mannose rich O-polysaccharide. LPSs possessing this type of O-antigen have been shown to have greater adjuvant activity than other LPS types (Ohta et al., 1985; Kido et al., 1985; Ohta et al., 1987; Yokochi et al., 1990; Yokochi et al., 1992). Chapter 4 showed how LPS containing mannose homopolymers could augment an antibody response to CGG in mice hyporesponsive to LPS. The failure of this LPS to induce the proliferation of B cells in splenocytes from C3H/HeJ mice suggests that a direct effect on B cells is not involved in its adjuvanticity. These observations are consistent with those of Ohta and colleagues (Ohta et al., 1985), who also suggested that LPS containing a mannose O-polysaccharide does not enhance B cell responses by inducing TLR4-independent B cell proliferation. Instead, it has been shown that an increase in adjuvant activity correlates with increased

complement fixation by linear mannan-possessing LPS (Yokochi et al., 1990). This may be an alternative mechanism for enhanced adjuvanticity in the absence of TLR4 signalling.

No T cell proliferation was observed for any of the samples except when stimulated with PMA and ionomycin. PMA is a phorbol ester that activates protein kinase C (PKC) which is important in many processes including controlling cell division. Phorbol esters activate PKC because they resemble diacylglycerol. Ionomycin is a calcium ionophore. It is used in conjunction with PMA because PKC can only induce cell proliferation in the presence of calcium ions.

T cells in C3H/HeJ and C3H/HeN splenocytes populations failed to proliferate when cultured with LPS. This result contrasts with findings from previous studies showing that *in vitro* stimulation of purified splenic T cells with LPS can induce their proliferation. This activity was shown to be polymyxin B sensitive, and T cells from C3H/HeJ mice failed to proliferate (McGhee et al., 1979, Vogel et al., 1983). These studies used isolated T cells, and it might be possible in these studies that contaminating cells such as B cells were proliferating in response to LPS. Although LPS has also been shown to stimulate T cells *in vivo* this was dependent on the presence of APCs and type 1 interferons (Tough et al., 1997). In this study, *Klebsiella* DNA failed to stimulate C3H/HeN and C3H/HeJ T cells from splenocyte populations *in vitro.* Although T cell stimulation by CpG DNA has been shown in *in vivo* studies, the effect was indirect and mediated by type 1 interferons (Sun et al., 1998).

In vitro studies presented in this thesis indicate that *Klebsiella* DNA has no effect on T cell proliferation.

Interestingly, both *Klebsiella* K52 and K55 CPS pool 1 failed to induce the proliferation of C3H/HeJ splenocytes, although stimulation of C3H/HeN splenocytes was observed. This suggests strongly that a TLR4 agonist, most likely LPS, in the *Klebsiella* CPS pool 1 induced B cell proliferation in the C3H/HeN mice. These results are entirely consistent with the data described in Chapter 4. *Klebsiella* CPS pool 1 has no adjuvant properties in LPS-hypresponsive mice. In contrast, *Klebsiella* CPS pool 2 could induce proliferation of B cells in a LPS- or TLR4-independent manner. This again was consistent with the presence of LPS-independent adjuvant activity within this pool as shown in Chapter 4 of this thesis.

Klebsiella CPS extracts that were protease digested and subjected to mild alkali treatment still induced significant proliferation of C3H/HeJ splenocytes suggesting that intact protein and alkali-sensitive lipids were not contributing to the activity. Samples subjected to harsher alkali treatment failed to induce proliferation of both C3H/HeN and C3H/HeJ splenocytes. The latter treatment would detoxify LPS (confirmed by the lack of B cell proliferation in C3H/HeN splenocytes), denature DNA and proteins, and de-O-acylate lipid components. The CPS is stable under these conditions but any lipid-linked anchors would be lost. The active component(s) is therefore relatively stable, resistant to protease and nuclease digestion and alkali conditions. It also appears to be heat and alcohol resistant as the sample was subjected to

ethanol precipitation and heating at a temperature of up to 100°C during the extract procedure. Although highly purified CPS of very high MW has no immunomodulatory effects the possibility of the presence of biologically active, smaller lipidated CPS in pool 2 has not been ruled out.

Profiles of cytokine release from C3H/HeJ splenocytes were also determined to be in accordance with the results of the proliferation assays. Very little cytokine production above the media only control was observed when C3H/HeJ splenocytes were cultured with LPS. Both K55 and K52 CPS extracts induced TNF-α, IFN-γ, MCP-1, IL-6 and IL-10. The production of cytokines was induced by components present in *Klebsiella* CPS pool 2 but not in pool 1. *Klebsiella* DNA at the concentration present in the CPS extracts induced TNF-α, IFN-γ, IL-6, and MCP-1, albeit at lower concentrations compared to the CPS extracts. This confirms that cytokine release induced by CPS extracts was not solely due to *Klebsiella* DNA stimulation. CpG DNA is a ligand for TLR9 and was expected to produce the above set of proinflammatory cytokines.

Interestingly no IL-12p70 was detected in supernatants from cells stimulated with either DNA or CPS extracts in this study. IL-12 is a proinflammatory cytokine which induces the production of IFN-γ. A large amount of IFN-γ was detected in the supernatants and therefore the absence of IL-12p70 was surprising. Biologically active IL-12 is a heterodimeric protein composed of p35 and p40 subunits (Stern et al., 1990). If more IL-12p40 is produced, homodimers form, which can bind to IL-12R, but fail to mediate a signal

thereby serving as a functional antagonist to IL-12p70 heterodimers (Mattner et al., 1993, Ling et al., 1995). Only the presence of IL-12p70 was assayed, so it was possible that there was an over production of IL-12p40 and homodimers were being formed. Cells stimulated with CPS extracts also produced high levels of IL-10, a negative regulator of IL-12 production, which could also explain the absence of IL-12. The production of IFN-γ was still observed, even when no IL-12p70 was detected. IL-18 is also a known IFN-γ inducing cytokine. It is generally thought that both IL-12 and IL-18 are required for IFN-γ production but a number of studies in the literature have suggested that IFN-γ can be induced by IL-18 in an IL-12 independent manner (Muller et al., 2001b, Xing et al., 2000, Wakatsuki et al., 2003). This possibility should be further investigated in the future.

Known TLR 2 ligands, Pam3Cys and MDP, were also included in the study. Bacterial lipoproteins are a family of proinflammatory cell wall components found in both Gram negative and Gram positive bacteria. The stimulatory activity of bacterial lipoprotein resides in its acylated amino terminus, and is mimicked by the synthetic Pam3Cys lipopeptide. Pam3Cys is known to induce the production of IL-1, IL-6 and TNF-α from macrophages (Muller et al., 2001a). In these studies, both TNF-α and IL-6 were produced by C3H/HeJ splenocytes. The presence of IL-1 was not determined. Very little IFN-γ was produced compared to the amounts induced by CPS extracts. MDP failed to have a dramatic effect on cytokine release. MDP is chemically synthesised and is the minimal essential structure of peptidoglycan, a constituent of bioactive cell walls from Gram negative and Gram positive

bacteria. Nagao and colleagues reported similar observations in that MDP failed to activate murine macrophages *in vitro* (Nagao et al., 1992, Nagao et al., 1990).

Klebsiella CPS extracts were shown to induce B cell proliferation in a mixed splenocyte population. Whether or not B cells are activated directly without the involvement of APCs is not known. It is possible that B cell proliferation is *via* cytokine production such as IL-6, which was shown to be present in the supernatant, from APCs. No T cell proliferation was observed, consistent with the absence of IL-2 in the supernatants of the cell cultures. No IL-12 was induced with CPS extracts but IFN-γ was produced. IFN-γ is predominantly produced by T cells, natural killer cells (NK), and NKT cells in response to infection, cytokines or mitogens. Evidence has shown the importance of IFN-γ in both acquired and innate immunity (Magram et al., 1996, Andersson et al., 1998). IFN-γ is a major proinflammatory cytokine because of its ability to activate macrophages and endothelial cells (Goodbourn et al., 2000, Dalton et al., 1993). It is important that adjuvants have the capacity to induce cytokines that can activate APCs. Adjuvants that stimulate APCs can enhance antigen presentation, increase cytokine production and costimulatory molecule expression such as CD80, CD86, and CD40 on APCs. TNF-α, a proinflammatory cytokine capable of inducing the maturation of DCs, was produced by cells stimulated with *Klebsiella* CPS extracts. Mature DCs migrate from the peripheral tissues to the draining lymph nodes, where they instruct the adaptive immune response by stimulating T lymphocytes. Both IL-6 and IFN-γ can induce immunoglobulin

class switching. The *in vitro* effects of *Klebsiella* CPS extracts on splenocytes have similarity to a known TLR2 agonist, Pam3Cys, but were not identical with respect to IFN-γ production.

Our results suggest that *Klebsiella* CPS extracts from K52 and K55 contain components that can activate immune cells via at least TLR2 and TLR4, but not TLR9. Stimulation with CPS extracts resulted in the production of major proinflammatory cytokines by splenocytes, and the proliferation of splenic B cells. Lack of availability of TLR or TLR-associated adapter protein knockout mice including TLR2 and MyD88 during the course of this thesis investigation has prevented further dissection of the underlying mechanism of their adjuvant activity. However these studies will be pursued when these mice become available. Since the compositions of *Klebsiella* CPS extracts have not been clearly defined, further investigations are needed in order to determine the identities of the immunostimulatory components in the extracts. However, our results have disproved the assumption that pure CPS is one of these components. In addition, this is the first study to indicate an immunomodulatory component or components from *Klebsiella* CPS extracts that appear to be extremely resistant to heat, protease, nuclease and alkali treatments. Further biochemical purification and immunological analysis will be required in order to determine whether or not the component(s) is a novel adjuvant candidate, and their mode of action.

Chapter Six

Results

Chapter Six

The role of complement in the humoral response to *Streptococcus pneumoniae* capsular polysaccharides

6.1 Introduction

The discovery of complement receptors on the surface of B cells was the first indication that complement has an important role in the induction of antibody responses (Lay and Nussenzweig, 1968; Bianco et al., 1970; Nussenzweig, 1974). Pepys and colleagues showed that the antibody response to T-dependent antigens was abrogated by transient depletion of C3 in mice with cobra factor venom (CVF) (Pepys, 1974). Other studies in guinea pigs (Ellman et al., 1971, Bottger et al., 1986), dogs (O'Neil et al., 1988), humans (Hazlewood et al., 1992) and mice (Fischer et al., 1996) established that genetic deficiencies in complement components C2, C3 or C4 resulted in reduced humoral responses. The conjugation of the C3d fragment of C3 to T-dependent and T-independent type II antigens has also been studied. Dempsey showed that fusing mouse C3d to hen egg lysozyme (HEN) resulted in HEN becoming 1,000 to 10,000 fold more immunogenic in mice compared to unmodified HEN (Dempsey et al., 1996). Test and colleagues showed that the same effect could be seen when C3d was coupled to a pneumococcal serotype 14 CPS, a T-independent type II antigen (Test et al., 2001). Thus, C3d acts as an adjuvant for antibody responses.

The effect of complement on B cell responses is mediated by complement receptor 1 (CD35) and 2 (CD21) which are found on the surface of both B cells and follicular dendritic cells (FDCs). Studies have been carried out using antibodies specific to CD21 and CD35 in mice. The administration of CD21-specific antibodies resulted in a diminished primary antibody response to T-dependent and T-independent antigens, whereas CD35-specific antibodies had less effect on the humoral response (Thyphronitis et al., 1991, Gustavsson et al., 1995, Heyman et al., 1990). Another study which involved the use of soluble CD21 resulting in reduced T-dependent responses in mice (Hebell et al., 1991), further highlighted the more significant role of CD21 in enhancing antibody responses compared to CD35.

A variety of methods have been used to dissect the role of complement in humoral responses, some which have already been described. A number of groups have generated mice that are deficient in CD21 and CD35 by gene disruption. CD21 and CD35 are encoded at a single locus (Cr2) in mice. Disruption of the CR2 locus abolishes expression of both CD21 and CD35. Several groups have independently generated Cr2 -/- mice (Ahearn et al., 1996, Molina et al., 1996, Croix et al., 1996), and all groups showed the humoral response to be diminished in their mutant mice, although the degree of impairment was not consistent. Ahearn and colleagues showed there was no antibody response in Cr2 -/- mice to bacteriophage ΦX174, not even when an adjuvant was administered (Ahearn et al., 1996). Although high antigen doses did induce a low antibody response, this was significantly reduced compared to wild type (WT) mice. Similar results were observed by

Croix and colleagues, in that mice that did not express CD21/CD35 on B cells failed to make an antibody response to (4 hydroxy-3-nitrophenyl)acetyl (NP)–keyhole limpet hemocyanin with alum adjuvant (Croix et al., 1996). Other independent studies showed the antibody response to sheep red blood cells (SRBC) was reduced compared to wild type mice to both low and high dose antigen (Molina et al., 1996), whereas the antibody response to NP conjugated to CGG was reduced in Cr2 -/- with low dose antigen, but increasing dose partially corrected this deficit (Chen et al., 2000).

There are two mechanisms that can account for the role of CD21 and CD35 in enhancing antibody responses. Firstly, co-ligation of the B cell receptor (BCR) with the complement complex (CD21/CD19/CD81) by C3 fragments (C3d) attached to antigen results in lowering of the threshold for B cell activation. Secondly, retention of the antigen-complement complex by FDCs in germinal centres via binding to CD21/CD35 enables B cells to access antigen (Carroll, 1998, Fang et al., 1998, Qin et al., 1998). Studies have been carried out to determine whether FDCs or B cells are important in the abrogated response to T-dependent antigens in Cr2 -/- mice. Fang and colleagues showed that a defective antibody response was still observed even when Cr2 -/- mice were reconstituted with Cr2 +/+ bone marrow suggesting an important role for FDCs (Fang et al., 1998). Other studies showed that mice lacking CD21/CD35 expression on B cells also failed to produce an antibody response (Croix et al., 1996). It is clear from these studies that expression of CD21 and CD35 on both B cells and FDCs is important in producing a good antibody response. Cr2 -/- mice have been

shown to be able to form germinal centres, although they are fewer in number and reduced in size. They also display accelerated loss of serum antibody and fewer long-lived antibody-forming cells (Chen et al., 2000).

Most studies have examined the role of complement in the humoral response to T-dependent antigens. Fewer studies have focussed on T-independent type II antigens and the role of complement in the response to these antigens still remains controversial. Early studies from Pepys showed no change in the antibody response to polyvinylpyrrolidone 360 in mice depleted of C3 (Pepys, 1974). In contrast Markham and colleagues showed that depletion of C3 abrogated the antibody response to pneumococcal serotype 14 polysaccharide but had no effect on the response to the sialic acid containing group B *Streptococcus* type III capsular polysaccharide (Markham et al., 1982). Other studies have shown a reduced IgM and IgG response to group B *Streptococcus* type III capsular polysaccharide in Cr2 -/- mice. This impairment was characterised by negligible uptake of Ag by FDCs and MZ B cells (Pozdnyakova et al., 2003). One explanation for the differences observed in the humoral response to group B Streptococcus type III capsular polysaccharide is that CVF depletes circulating C3 but is unlikely to affect the local production of C3 by myeloid cells in lymphoid compartments. Studies have shown that local synthesis of C3 is important and can enhance antibody responses to protein antigens independent of C3 in the circulation (Verschoor et al., 2001, Fischer et al., 1998).

6.2 Objectives

The role of complement in the antibody responses to physiological TI-2 antigens remains unclear. Most studies have focussed on model TI-2 antigens, such as DNP-Ficoll. CPSs from Gram positive *S. pneumoniae* are commercially available as a 23-valent polysaccharide vaccine and usually devoid of LPS contamination that is commonly found in CPS preparations from *Klebsiella pneumoniae* and other Gram negative bacteria. This allowed us to study a large set of structurally diverse and clinically relevant TI-2 antigens and to establish the role of mouse complement receptors 1 and 2 in anti-CPS responses by comparing the responses of C57BL/6 and Cr2-/- mice.

6.3 Results

6.3.1 Expression of CD21/CD35 in Cr2 -/- and C57BL/6 mice.

The level of expression of complement receptors CD21/CD35 in wild type (C57BL/6) and Cr2-/- mice was compared by FACs. As shown in Figure 6.1 Cr2-/- mice used in this study have a much reduced level of expression but not a complete absence of CD21/CD35. This is consistent with other studies which have shown that this particular line of Cr2-/- mice express a truncated form of CD21/CD35 due only exons 9 and 10 being spliced out (Hasegawa et al., 2001). Although there appear no major differences in antibody responses between this and other lines of Cr2-/- mice, it was nevertheless important that studies in Cr2-/- mice were compared to those in C3-/- mice (complement component 3-deficient mice).

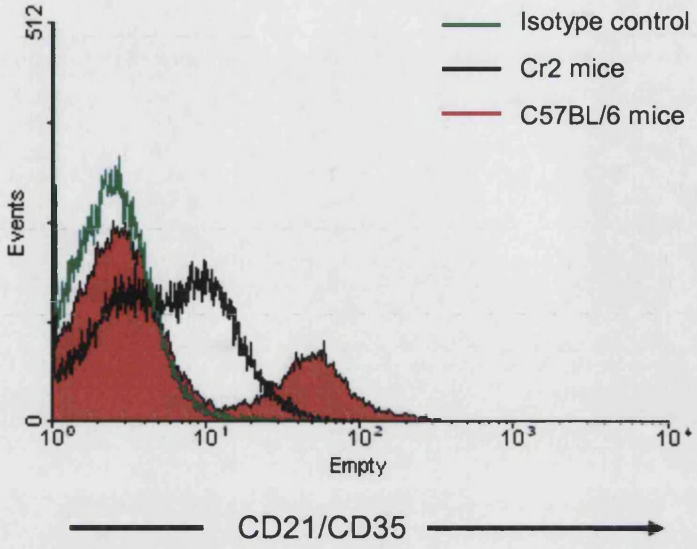


Figure 6.1 Expression of CD21/CD35 on naïve splenocytes from C57BL/6 and Cr2-/- mice.

Splenocytes were harvested and labelled with an antibody to CD21/CD35 and appropriate isotype controls were used. These data are representative of 3 separate experiments.

6.3.2 The presence of natural IgM antibodies in naïve mice to *Streptococcus pneumoniae* CPSs.

Natural IgM antibodies are an important part of innate immunity. Some of these antibodies recognise microbial carbohydrates. Serum from mice has been shown to contain these natural antibodies. It is therefore important to assess the presence of natural IgM in naïve C57BL/6 and Cr2-/- mice to *S. pneumoniae* capsular serotypes. Blood was taken from naïve mice and analysed by ELISA. Figure 6.2 shows a comparison of IgM levels in naïve C57BL/6 and Cr2-/- mice to different capsular serotypes. IgM levels to 2, 9V, 9N, 14, 18C, 19A, 19F, and 23F in C57BL/6 were significantly higher than in Cr2-/- mice. For CPSs 1, 3, 4, and 6B there was no significant difference in IgM levels in naïve C57BL/6 and Cr2-/- mice. The background IgM levels for the pneumococcal cell wall polysaccharide (CW-PS), a common contaminant of pneumococcal preparations, were also determined in Cr2-/- and C57BL/6 mice. The IgM levels were found to be significantly reduced in Cr2-/- mice compared to C57BL/6 mice.

6.3.3 The humoral response to *Streptococcus pneumoniae* CPS in wild type mice

6.3.3a Dose response

Very few studies have investigated the humoral response to *S. pneumoniae* CPSs in mice. Before investigating the immune response in Cr2 -/- the responses in wild type mice needed to be established. Studies that have been carried out have shown that there are several parameters that can influence the immune response to polysaccharide antigens; these include

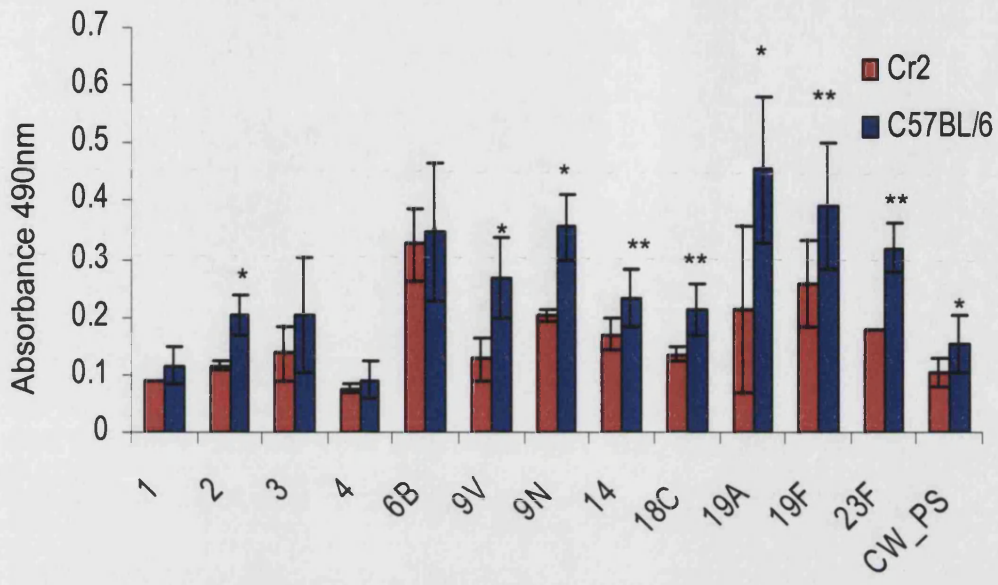


Figure 6.2 A comparison of serum IgM levels in naïve C57BL/6 and Cr2-/- mice to *Streptococcus pneumoniae* capsular polysaccharides.

Sera from naïve C57BL/6 and Cr2-/- mice were analysed for IgM to a range of pneumococcal serotypes by ELISA at 1 in 200 dilution and preabsorbed with 100ug/ml CW-PS. IgM levels to CW-PS were determined at a 1 in 200 dilution without preabsorption. Goat anti-mouse IgM (μ chain specific) HRP conjugate was used to detect IgM. OPD was used as a substrate. Data represents the mean of six to ten mice ± s.e.m.(Cr2 ≠C57/Bl6; *, $p<0.05$; **, $p<0.01$).

genetic background, age of mice, antigen dose and route of administration. C57BL/6 mice aged 8 -12 weeks were immunised by the intra peritoneal route with a mixture of *S. pneumoniae* CPSs 19A, 19F, 6B, and 14 at a range of doses (0.1 - 5µg of each CPS). Mice were bled 7 days post immunisation and then serum was analysed for IgM by ELISA. The dose response is shown in Figure 6.3. For each dose the absorbance is compared. Any increase in absorbance reflects an increase in IgM levels in the serum. For all CPSs tested there was clearly an increase in IgM levels when dose was increased. Serum taken 21 days post immunisation gave similar results (data not shown).

6.3.3b Cell Wall polysaccharide preabsorption

The purified pneumococcal CPSs obtained from either NIBSC or Statens Serum Institute may contain a certain amount of CW-PS which is a major constituent of the pneumococcal cell wall (Musher et al., 1990). When mice are immunised with purified pneumococcal CPSs they are also immunised with CW-PS. It is therefore important that CW-PS is used to absorb mouse serum to remove antibodies to this polysaccharide when assessing antibodies to pneumococcal capsular polysaccharide antigens by ELISA. The presence of natural IgM to CW-PS as shown in Figure 6.2 means that naïve mouse serum should also be pre-absorbed with CW-PS. Absorption was carried out with 100µg/ml of CW-PS for one hour at RT at a 1 in 50 dilution of serum in PBS with 0.05% (w/v) Tween. CW-PS coated ELISA plates were used to verify that all CW-PS specific antibodies were prevented from binding to CW-PS by the absorption technique. This method has been

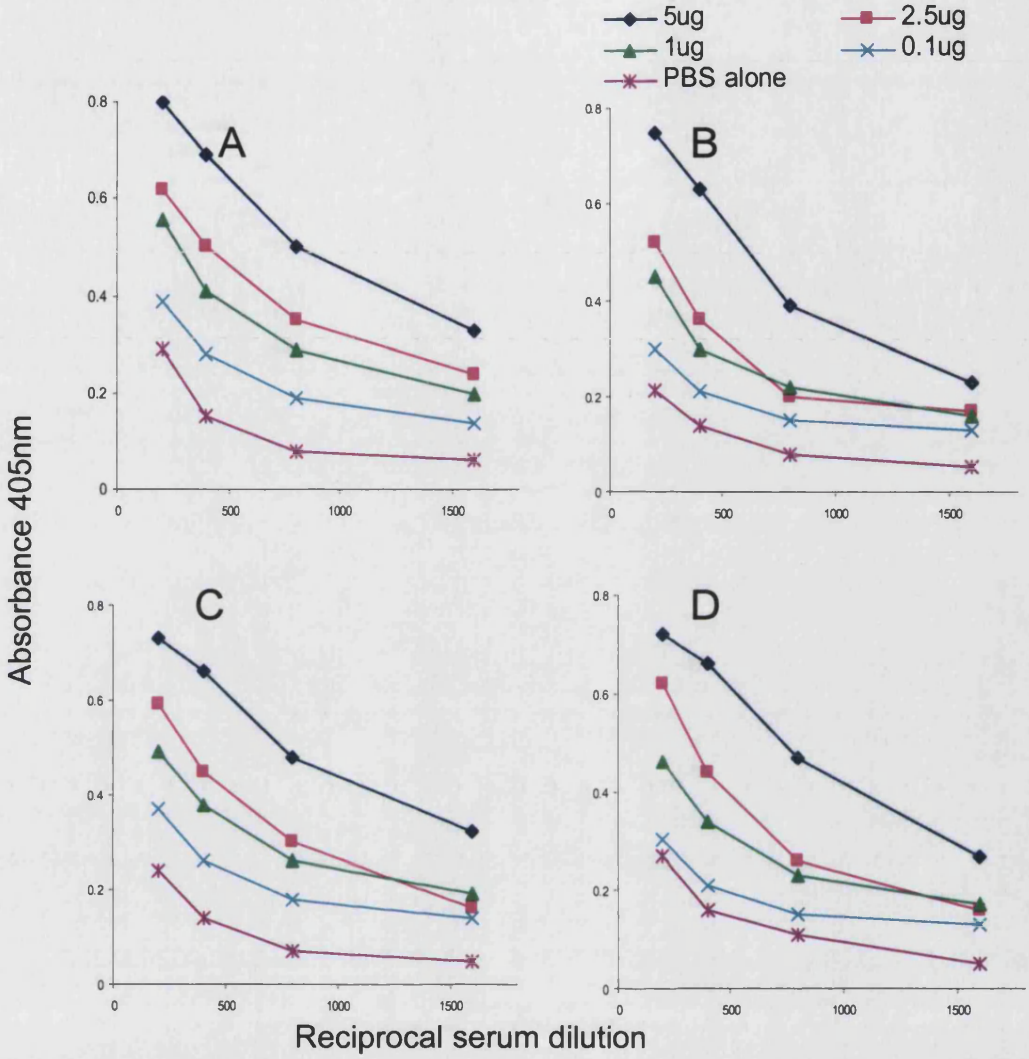


Figure 6.3 Comparison of serum IgM response (7 days post immunisation) to *Streptococcus pneumoniae* capsular polysaccharides types 19A, 19F, 14 and 6B at different doses in C57BL/6 mice.

Groups of three C57BL/6 mice were immunised i.p with a mixture of pneumococcal CPS serotypes (19A, 19F, 14 and 6B) at a range of doses (0.1 – 5μg of each CPS in sterile PBS) or with PBS alone. Blood was taken 7 days post immunisation and pooled and then analysed for IgM levels to serotypes 19A (A), 6B (B), 19F (C) and 14 (D) by ELISA. Mouse IgM was detected by goat anti-mouse IgM (μ chain specific) HRP conjugate. ABTS was used as a substrate. IgM levels were assessed by absorbance readings at 405nm wavelength. These data are means of duplicate wells and are representative of two separate experiments.

used by others but at a reduced concentration of CW-PS for absorption (Musher et al., 1990; Aaberge et al., 1993; Sankilampi et al., 1996), but our studies have shown that CW-PS concentrations less than 100μg/ml are not consistent at neutralising the serum of CW-PS specific antibodies.

6.3.3c Effect of Booster

The effect of a booster immunisation was also investigated. Mice were immunised as before with a 5μg mix of CPSs 19A, 19F, 6B and 14 and then again at day 14. Mice were bled 7 days before and after booster immunisation. Figure 6.4 shows the effect of a booster on the IgM response to CPSs 19A and 19F. There was little difference in absorbance levels before and after administration of a booster immunisation. This was also the case for the other pneumococcal CPSs and at all doses employed (data not shown). These results are entirely consistent with what is generally known about polysaccharide antigens. Polysaccharide antigens do not induce immunological memory, which is a prerequisite for enhancement of an immune response by a booster immunisation. It is therefore for this reason there was no effect on the IgM levels present in the serum of mice before and after booster immunisation. This has been shown to be the case for pneumococcal CPSs (Baker et al., 1971; Aaberge et al., 1993).

6.3.3d Longevity of IgM response

The longevity of the IgM response to pneumococcal CPSs 19A and 19F are shown in Figure 6.5. Mice were bled 6 months post immunisation and serum was analysed for IgM and compared to day 7 and 21 levels. All time points

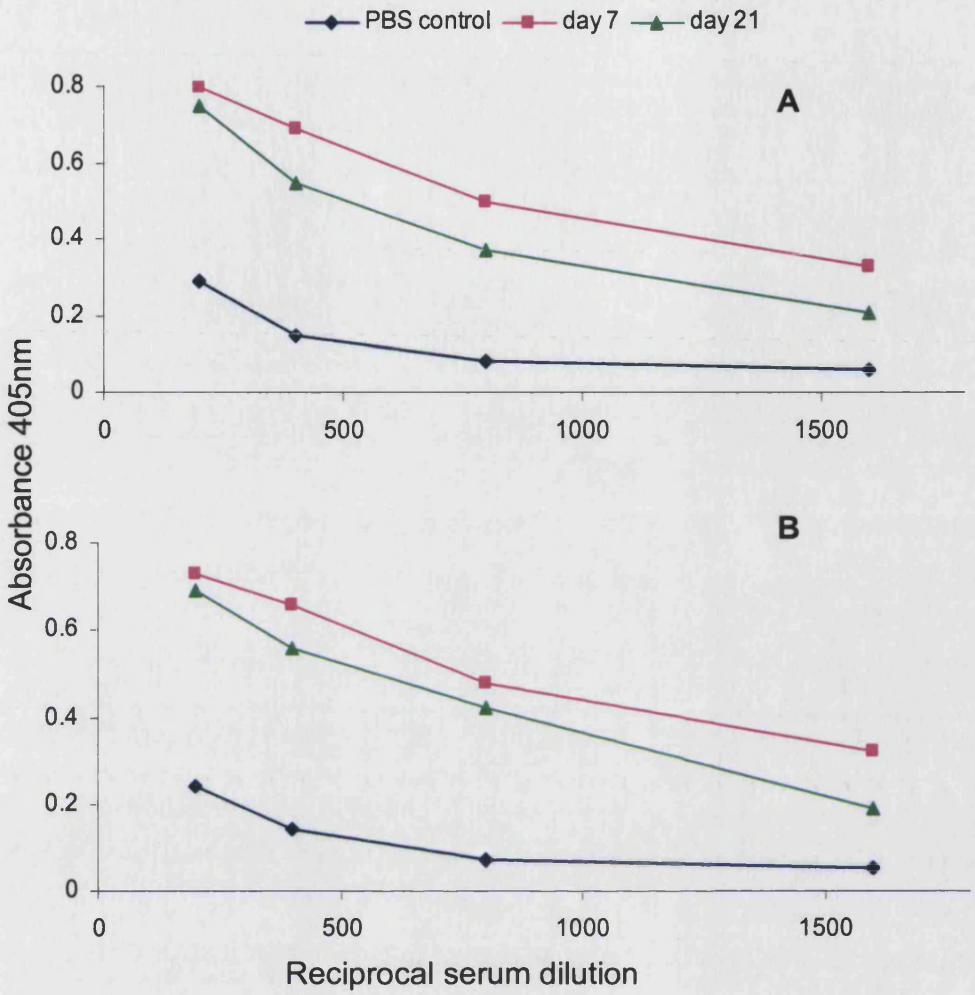


Figure 6.4 Comparison of serum IgM responses to *Streptococcus pneumoniae* capsular polysaccharides 19A and 19F before and after booster immunisation in C57BL/6 mice.

Groups of three C57BL/6 mice were immunised with a mixture of pneumococcal CPS serotypes 19A, 19F, 6B and 14 (5μg of each) or PBS only. A booster was given at day 14 (same as the first immunisation). Mice were bled at day 7 and day 21, serum was pooled and then analysed by ELISA using a goat anti-mouse IgM (μ chain specific) HRP conjugate and ABTS substrate. IgM levels were assessed by absorbance at 405nm wavelength. Only 19A (A) and 19F (B) are shown. These data are means of duplicate wells and are representative of two separate experiments.

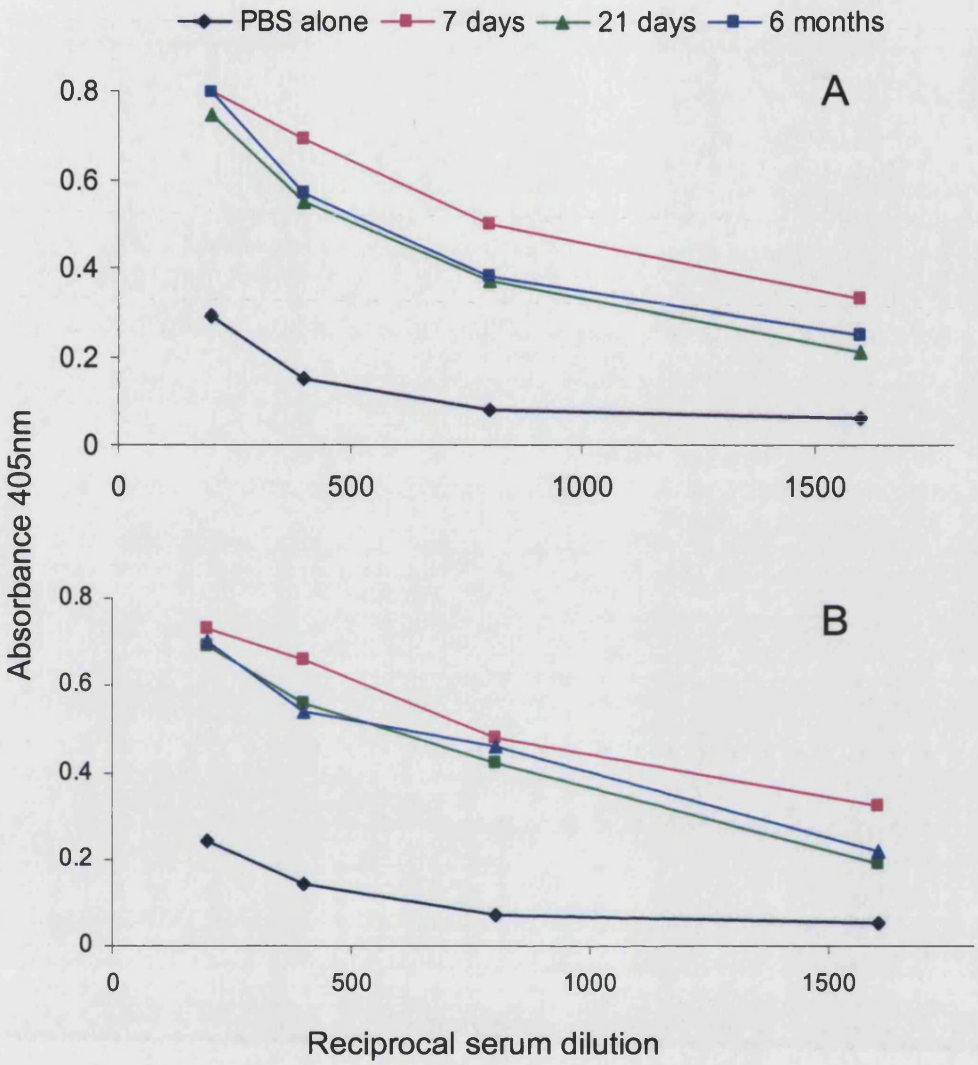


Figure 6.5 Long term serum IgM response to *Streptococcus pneumoniae* capsular polysaccharides types 19A and 19F in C57BL/6 mice.

Groups of three C57BL/6 mice were immunised i.p with a mixture of 5μg of pneumococcal serotypes 19A, 19F, 14 and 6B or PBS only. Mice were bled 7days, 21 days and 6 months after immunisation with the four polysaccharides. A booster was given at day 14 (same as the first immunisation). Serum was pooled and analysed by ELISA, only 19A (A) and 19F (B) are shown. IgM levels were assessed by absorbance at 405nm wavelength. These data are means of duplicate wells and are representative of 2 separate experiments.

have very similar absorbance values, which indicate that antibody levels are maintained for at least 6 months post immunisation. This was also the case for pneumococcal CPSs 6B and 14 (data not shown). Other studies in BALB/c and CBA/J mice have also shown that the antibody levels to pneumococcal CPSs are maintained for up to five months (Aaberge et al., 1993).

The antibody response to pneumococcal CPSs 19A, 19F, 6B and 14 was investigated in BALB/c mice to determine whether the kinetics of the response were different for this mouse strain. A dose response 7 days post immunisation is shown in Figure 6.6. There is an absorbance increase for all CPSs compared to PBS control, but doses between 0.1 and 5μg have no effect on the magnitude of the antibody response. The dose dependent response that was observed in C57BL/6 mice (Figure 6.3) is not applicable to BALB/c mice. A booster immunisation was also given and resulted in no effect on the magnitude of the antibody response (data not shown).

6.3.4 A comparison of serum IgM responses to *Streptococcus pneumoniae* CPSs in C57BL/6 and Cr2-/- mice.

To compare the serum IgM response to CPSs in C57BL/6 (H-2^b) and Cr2-/- (H-2^b) mice, mice were immunised i.p with 5μg of each CPS. From the dose response data generated (Figure 6.3) 5μg of CPS gave a good detectable response for all of the CPS tested. For these experiments mice were immunised with individual CPS rather than a mixture of CPSs. Many previous studies have immunised mice with many different capsular serotypes at the same time to study the antibody responses to different CPSs

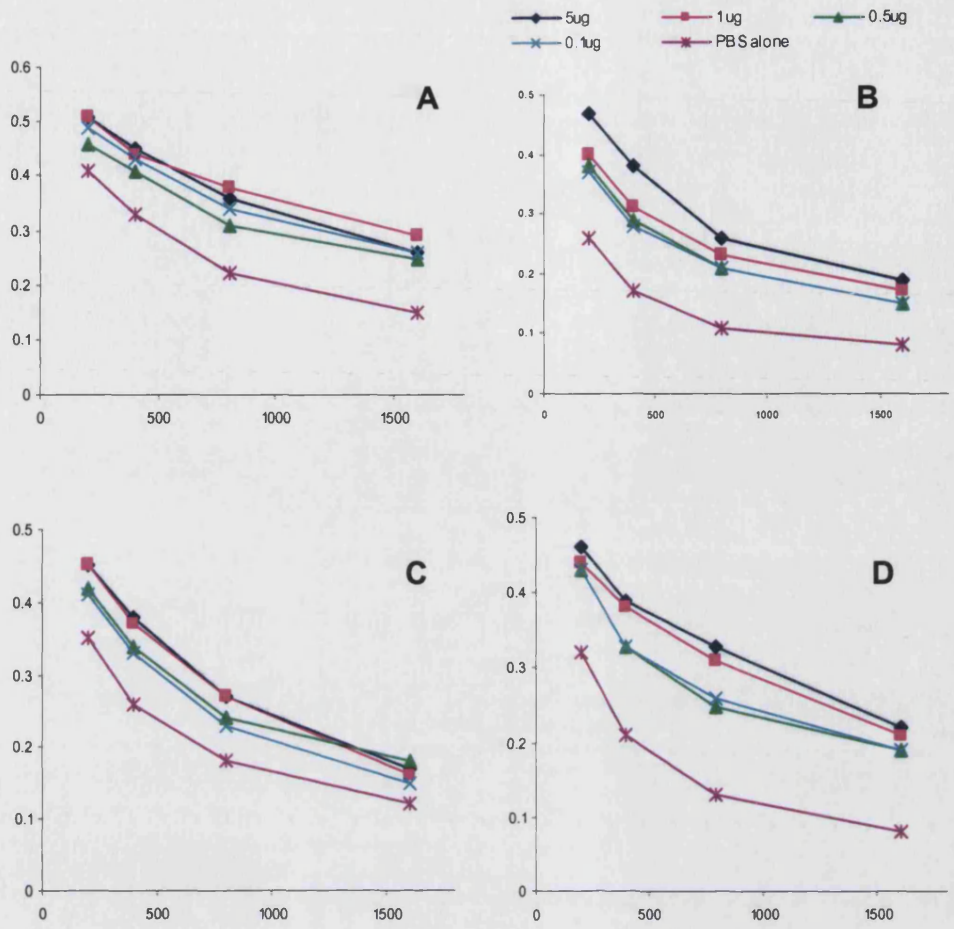


Figure 6.6 Comparison of serum IgM responses (7 days post immunisation) to *Streptococcus pneumoniae* capsular polysaccharides types 19A, 19F, 14 and 6B at different doses in BALB/c mice.

Groups of three BALB/c mice were immunised i.p with a mixture of pneumococcal CPS serotypes (19A, 19F, 14 and 6B) at a range of doses (0.1 – 5μg of each CPS in sterile PBS) or PBS alone. Blood was taken 7 days post immunisation and pooled and then analysed for the IgM to serotypes 19A (A), 6B (B), 19F (C) and 14 (D) by ELISA. IgM was detected with goat anti-mouse (μ chain) HRP conjugate using ABTS as a substrate. Absorbance was measured at 405nm. These data are means of duplicate wells and are representative of 2 separate experiments.

(Aaberge and Lovik, 1996, Aaberge et al., 1993), which is the method applied to Section 6.3.3 in this chapter. An extension to this work was to determine the specificity of the IgM response to each CPS. Results (from Dr. S. Zamze, Edward Jenner Institute for Vaccine Research, U.K.) have shown that the IgM produced after immunisation is not always specific to the immunising CPS. For example, mice immunised with 23F not only produced an antibody response to 23F but also to a range of pneumococcal CPSs (Figure 6.7). This was also the case when other CPSs were used to immunise mice (data not shown). For this reason it was decided that further experiments would be carried out using only single CPS immunisations.

Twenty one days after immunisation mice were bled and serum was assessed for IgM levels for each mouse. Figure 6.8 shows a comparison of IgM levels in C57BL/6 and Cr2-/- mice to pneumococcal CPSs after immunisation with either CPS or PBS only. These data are representative of three independent experiments. It is important that when comparing the response of both groups of mice that PBS-immunised mice are included. As shown in Figure 6.2 there was a significant difference between levels of IgM in naive Cr2-/- and C57BL/6 mice. It was for this reason that the difference between PBS-immunised and CPS-immunised mice for both Cr2-/- and C57BL/6 was compared rather than just the CPS-immunised mice.

The data shown in Figure 6.8 represents the absorbance values for sera at a 1 in 200 dilution. Initial experiments as described in this chapter were carried out using pooled sera from 3 mice and end point titre dilutions. However, on

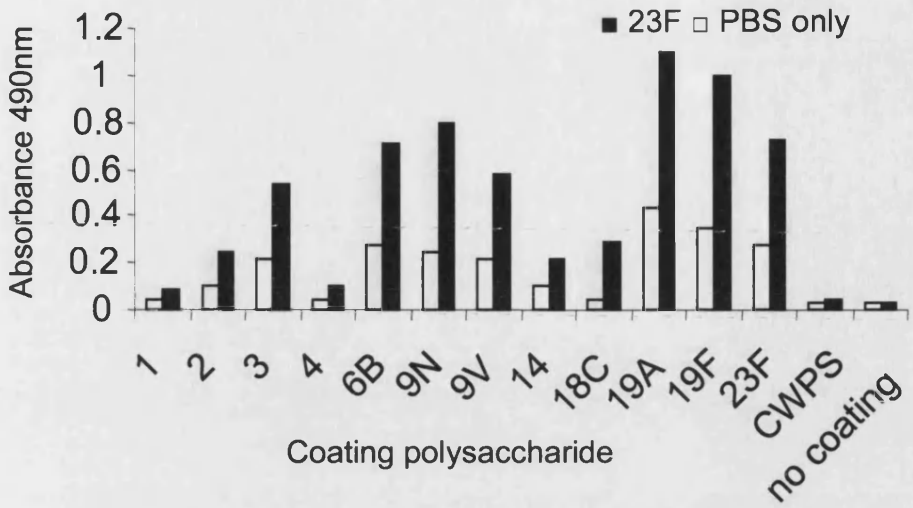
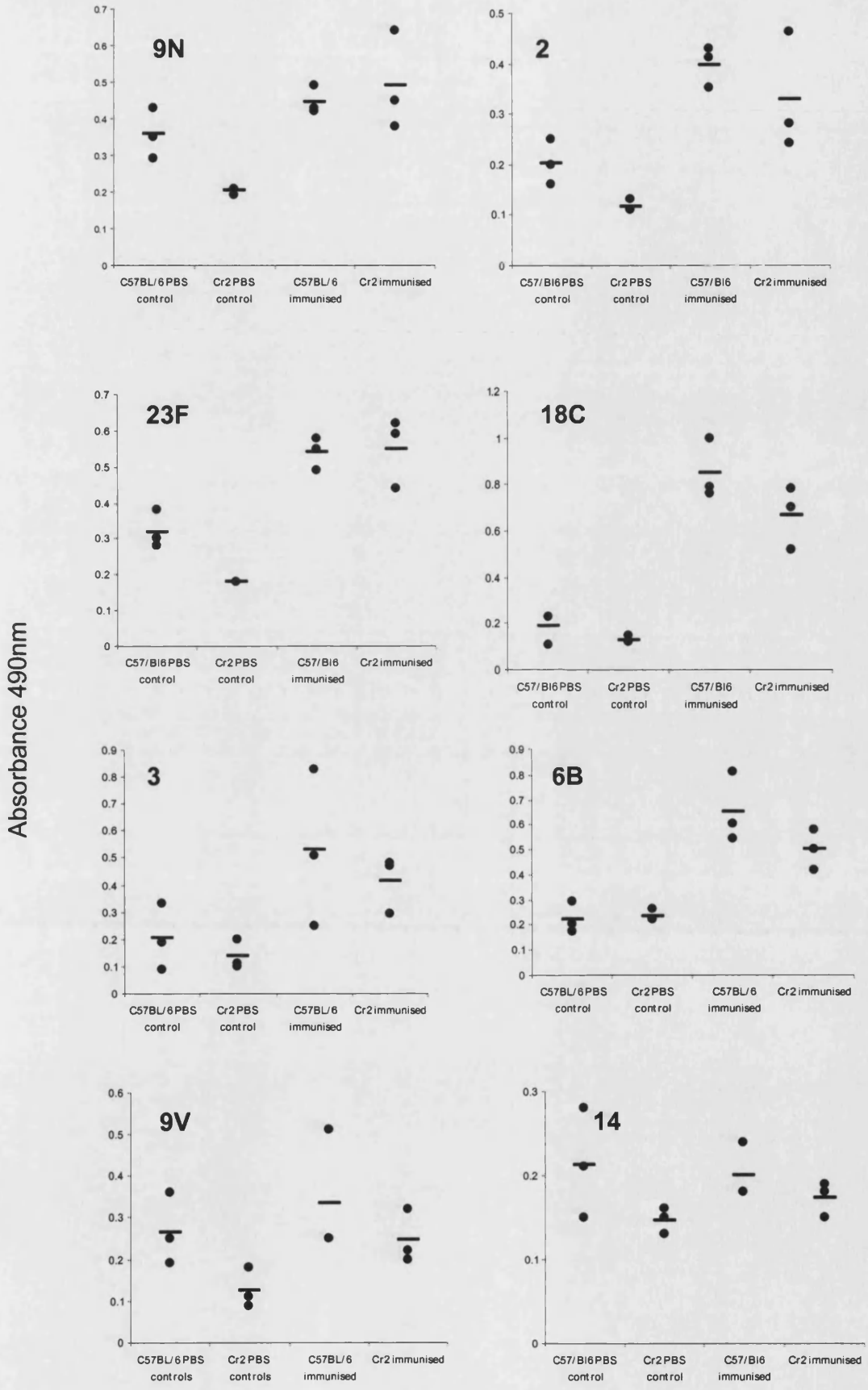


Figure 6.7 The binding of serum IgM to pneumococcal CPSs after immunisation of C57BL/6 mice with CPS serotype 23F.

Groups of three C57BL/6 mice were immunised with either 5μg of 23F (■) or PBS only (□). Mice were bled 21 days post immunisation and serum was collected and pooled. Serum was analysed at a 1 in 200 dilution (preabsorbed with CW-PS 100μg/ml) for IgM to a range of pneumococcal CPSs by ELISA (Section 2.2.4e). These data are means of duplicate wells and are representative of 3 separate experiments.


Absorbance 490nm
9N
C57BL/6PBS control
Cr2PBS control
C57BL/6 immunised
Cr2immunised
2
C57/Bl6PBS control
Cr2PBS control
C57/Bl6 immunised
Cr2immunised
23F
C57/Bl6PBS control
Cr2PBS control
C57/Bl6 immunised
Cr2immunised
18C
C57/Bl6PBS control
Cr2PBS control
C57/Bl6 immunised
Cr2immunised
3
C57BL/6PBS control
Cr2PBS control
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6B
C57BL/6PBS control
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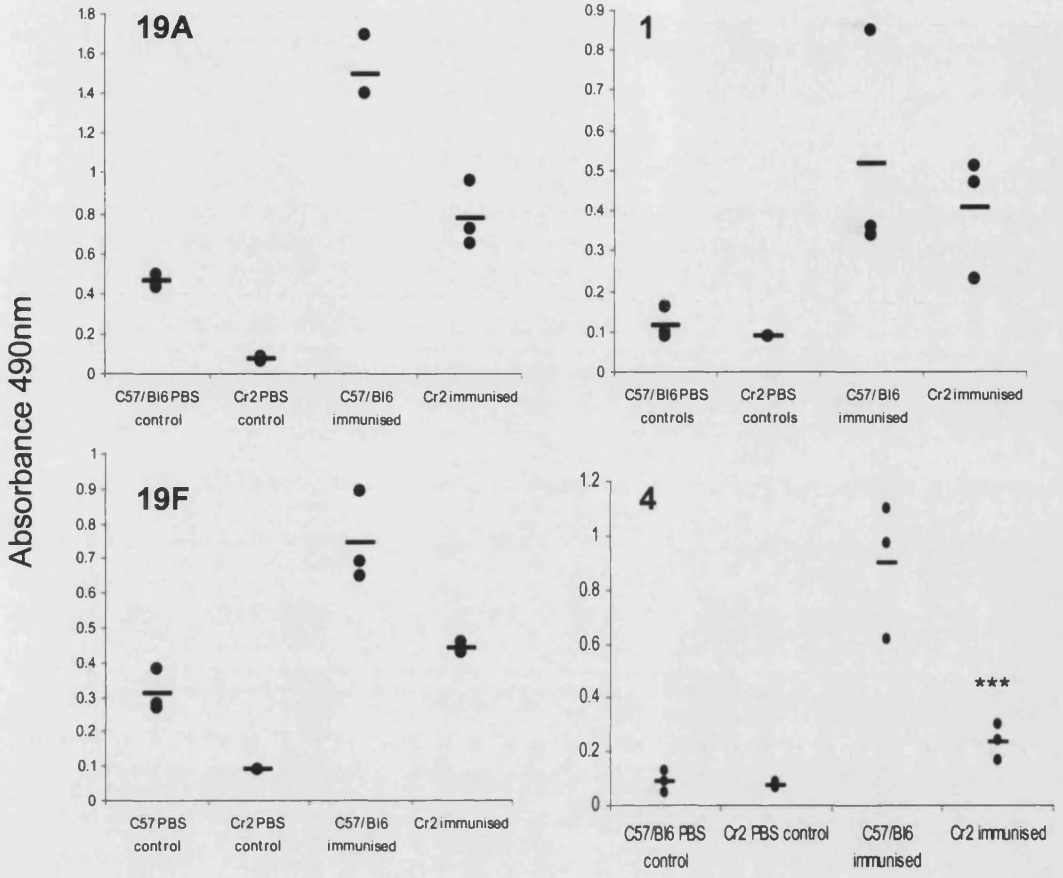

Figure 6.8 A comparison of serum IgM responses to *Streptococcus pneumoniae* capsular polysaccharides 9N, 2, 23F, 18C, 3, 6B, 9V, 14,19A, 1, 19F, and 4 in C57BL/6 and Cr2-/- mice.

Groups of three C57BL/6 and Cr2-/- mice were immunised by the intra peritoneal route individually with 5μg of pneumococcal CPS serotypes 9N, 2, 23F, 18C, 3, 6B, 9V, 14, 19A, 1, 19F and 4 or with PBS only. Mice were bled 21 days post immunisation and serum from each mouse was analysed for IgM to the CPS by ELISA at a 1 in 200 dilution of mouse serum. Goat anti-mouse IgM HRP conjugate was used to detect IgM. OPD was used as a substrate. Levels of IgM were assessed by absorbance at 490nm wavelength. Each mouse is shown by (•) and the mean of each group of mice is shown by (—). These data are means of duplicate wells from one representative study out of three. Significant differences are indicated as *** p=0.003.

examining the response from individual mice we noted considerable variation. Variation among mice in response to polysaccharide appears to be normal (Pozdnyakova et al., 2003). In order to interpret the results and allow statistical analysis it was decided the best approach would be to assay the sera from individual mice using two dilutions with absorbance values falling in the middle part of the titration curve. This approach for the analysis of anti-CPS antibody responses by ELISAs has been used as commonly as end point titres. In all cases where comparisons of titration curves from pooled sera and single readings from individual mice were carried out, the result, with respect to the difference in C57BL/6 and Cr2-/- mice, was the same. It was therefore decided that absorbance for a single dilution of serum provided the correct conclusions.

For all of the pneumococcal CPSs explored only capsular serotype 4 gave a significantly reduced response in the Cr2-/- mice compared to C57BL/6 mice. This indicates a complement dependent antibody response to serotype 4 CPS. The other CPSs vary in the magnitude of the response. Capsular serotype 14, when immunised alone, resulted in no IgM response above naïve levels. This was also the case when the dose was increased to 10μg (data not shown). The other CPSs gave IgM responses in C57BL/6 and Cr2-/- mice with the responses to both 9V and 9N being very low. Independent studies have also been carried out in C3-deficient mice (C3-/-) in our group. The results of these studies are consistent with those obtained with Cr2-/- mice.

The humoral response to pneumococcal CW-PS in C57BL/6 and Cr2-/- mice was also determined. Mice were immunised with 5μg of CW-PS and serum IgM and IgG levels were determined 7 and 21 days post immunisation. At day 7 there was consistently a very poor antibody response to CW-PS, but at day 21 the response was varied in C57BL/6 and Cr2-/- mice (data not shown). Three experiments were carried out in total, two with Cr2-/- mice and one with C3-deficient mice. Two experiments showed there was a good response in C57BL/6 mice but none in Cr2-/- mice. One experiment showed there was no difference in the IgM response in C57BL/6 and Cr2-/- mice. No serum IgG was found in any of the mice studied.

The model T-independent type II antigen DNP- ficoll was also included in the studies for comparison. Haas and colleagues showed that the IgM response in Cr2-/- to DNP-ficoll was normal compared to wild type mice, but the IgG response was significantly reduced (Haas et al., 2002). Figure 6.9 shows the IgM and IgG responses to DNP-ficoll. The IgM response was normal, but IgG response was significantly reduced in Cr2-/- mice. Our results expressed in absorbance values for DNP-Ficoll antibody responses are consistent with the literature.

Effect of dose on the complement dependent IgM response to type 4

Preliminary studies have been carried out to look at the effect of dose on the complement dependent response to capsular serotype 4. It was shown by Chen and colleagues that increasing dose of a protein antigen can overcome complement dependency (Chen et al., 2000). Figure 6.10 shows a

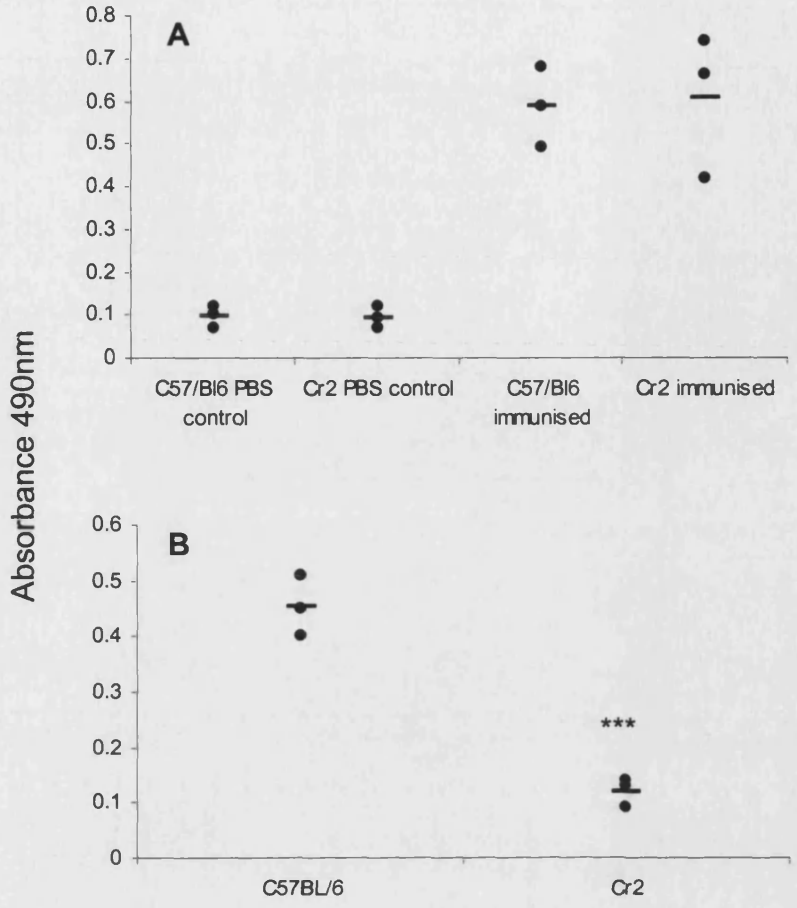

Figure 6.9 A comparison of serum IgM and IgG responses to DNP-ficoll in C57BL/6 and Cr2-/- mice.

Groups of three C57BL/6 and Cr2-/- mice were immunised with 5μg of DNP-ficoll. Mice were bled at 21 days post immunisation, then serum was analysed for (A) IgM and (B) IgG by ELISA. IgM was detected in individual mice at 1 in 200 dilution of serum for IgM and 1 in 100 dilution for IgG with goat anti-mouse IgM HRP conjugate. Individual mice are indicated by (•) and the mean of a group of 3 mice is shown as (—). These data are the means of duplicate wells from one representative study out of two (second study in C3 deficient). Significant differences are indicated as ** p = 0.001.

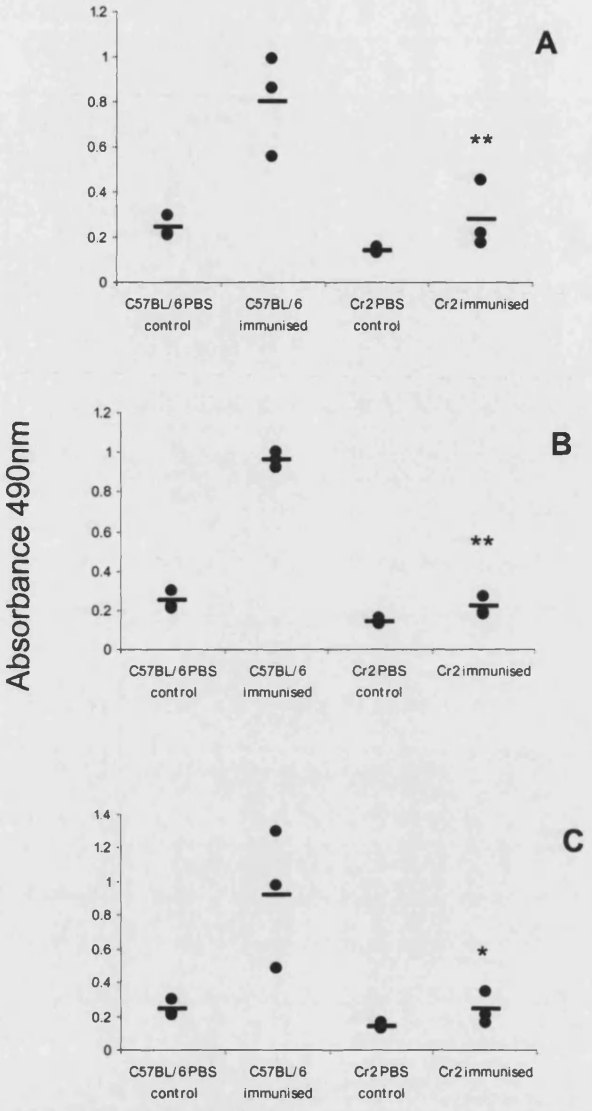

Figure 6.10 A comparison of serum IgM responses to different doses of *Streptococcus pneumoniae* capsular serotype 4 in C57BL/6 and Cr2-/- mice.

Groups of three C57BL/6 and Cr2-/- mice were immunised by the intra peritoneal route individually with either 5μg (A), 15μg (B), and 25μg (C) of pneumococcal serotype 4 or PBS only. Mice were bled 21 days post immunisation and serum was analysed by ELISA for the presence of IgM to serotype 4. Goat anti-mouse IgM (μ chain specific) HRP conjugate was used to detect IgM. OPD was used as a substrate. Levels of IgM were assessed by absorbance at 490nm wavelength. Each mouse is shown by (•) and the mean of each group of mice is shown by (—). These data are means of duplicate wells. (A) is one representative study out of five conducted. (B) and (C) are data from one preliminary study. Significant differences are indicated as * $p < 0.05$ and ** $p<0.005$.

comparison of IgM responses in C57BL/6 and Cr2-/- mice after immunisation with 5μg, 15μg, and 25μg of serotype 4. For all three doses there was a significant reduction in the response of Cr2-/- mice compared to C57BL/6 mice. Increasing dose did not overcome the complement dependent IgM response of capsular serotype 4, although it must be noted that these data were from a single experiment and the maximum dose was 25μg. It is therefore important that before any conclusions are made, that this work is repeated and possibly dose range is extended to include greater doses.

The effect of co-immunisation of CPSs on the IgM response in Cr2-/- mice

The IgM response to co-immunised CPSs was also investigated. Mice were co-immunised with 19A, 19F, 6B, and 14. Figure 6.11 shows a comparison of IgM levels in Cr2-/- and C57BL/6 mice to 19A, 19F, 6B, and 14. The absorbance levels in the Cr2-/- mice are significantly reduced compared to C57BL/6 mice. Interestingly when these CPS were immunised individually the IgM response was not complement dependent. Preliminary studies have been carried out to determine if the same response was observed with a different combination of fours CPSs. CPSs 9V, 9N, 18C and 23F were used to immunise mice and both Cr2-/- and C57BL/6 mice responded the same to the CPSs (data not shown).

6.3.5 A comparison of serum IgG responses to *Streptococcus pneumoniae* CPSs in C57BL/6 and Cr2-/- mice.

The IgG response in C57BL/6 and Cr2-/- mice to pneumococcal CPSs and DNP-ficoll was investigated. No IgG was detected in mice that were

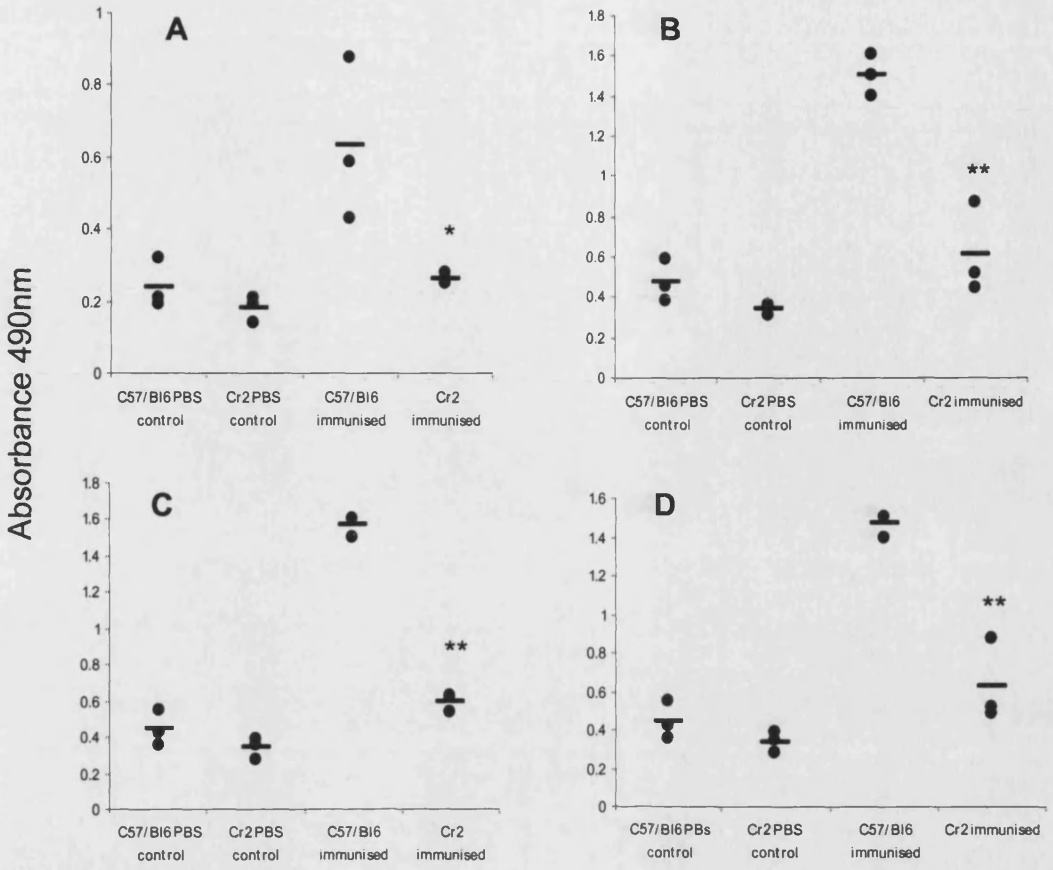

Figure 6.11 A comparison of serum IgM levels in C57BL/6 and Cr2-/- mice immunised with a mixture of *Streptococcus pneumoniae* capsular polysaccharides 14, 19A, 19F and 6B

Groups of three C57BL/6 and Cr2-/- mice were co-immunised (i.p) with 5μg each of pneumococcal serotypes 14, 19A, 19F and 6B or PBS alone. Blood was taken 21 days post immunisation and then analysed for IgM levels to serotypes 14 (A), 19F (B), 19A (C) and 6B (D) by ELISA at 1 in 200 dilution of mouse serum. IgM was detected by goat anti-mouse IgM (μ chain specific) HRP conjugate. OPD was used as a substrate. Absorbance at 490nm wavelength was used to assess IgM levels. Each mouse is shown by (•) and the mean of each group is shown by (—). These data are means of duplicate wells from one representative study out of two. (* $p< 0.05$; ** $p<0.02$)

immunised with CPSs 1, 2, 3, 6B, 9V, 9N, 18C, 19F, or 23F (data not shown). Previous studies have shown an absence of an IgG response to pneumococcal CPSs in mice when immunised with polysaccharide alone (Aaberge et al., 1993, Aaberge and Lovik, 1996, Test et al., 2001). However, IgG was found in some of the mice immunised with CPSs 4, 19A and 14. Figure 6.12 shows a comparison of IgG responses in Cr2-/- mice and C57BL/6 to serotypes 19A and 14. For both CPSs the IgG response is completely complement receptor dependent as shown by the absence of a response in the Cr2-/- mice. Even though there is an IgG response in C57BL/6 mice this is very variable, only two out of the three mice have an IgG to 19A response (Figure 6.12A).

The specificity of the IgG response to these polysaccharides was also determined. It has been shown that some batches of the 23-valent polysaccharide vaccine contain protein contaminants including pneumococcal surface protein A (PspA) and pneumococcal surface adhesion A (PsaA) (Yu et al., 2003). The IgG response could therefore be to a protein in the pneumococcal preparations. To investigate the presence of protein in our preparations, the pneumococcal CPSs were analysed by gel filtration chromatography on a TSK G5000 column with PBS at 40°C. Figure 6.13 shows a gel filtration profile for serotype 9V. This was representative of the other serotypes. The CPS eluted as a single peak around 30 mins as shown by refractive index indicating a molecular weight of approximately 2000 kDa. The 280nm trace, which indicates the presence of protein within the preparation, is very low. The pneumococcal CPSs were quantified for protein

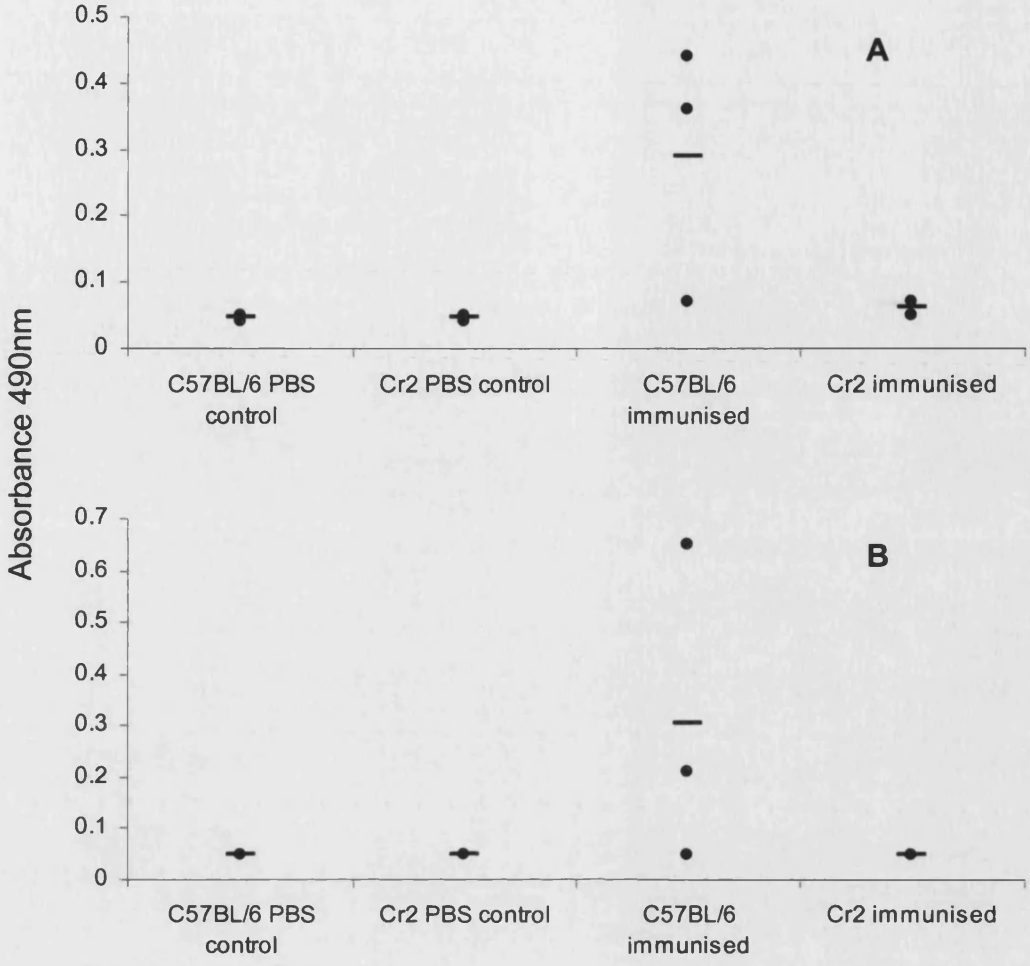

Figure 6.12 A comparison of serum IgG responses to *Streptococcus pneumoniae* capsular polysaccharides types 19A and 14 at day 21.

Groups of three C57BL/6 and Cr2-/- mice were immunised (i.p) with either 5μg of pneumococcal serotype 19A and 14 or PBS control. Mice were bled 21 days post immunisation and then serum was analysed for IgG responses to 19A (A) and 14 (B) by ELISA. IgG was detected by goat anti mouse IgG HRP conjugate for individual mice at a 1 in 200 dilution (A) and 1 in 50 dilution (B) of mouse serum. Absorbance at 490nm wavelength was used to assess IgG levels. Individual mice are indicated by (•) and the mean of a group of mice is shown by (—). These data are means of duplicate wells from one representative study out of two (A) and three (B)

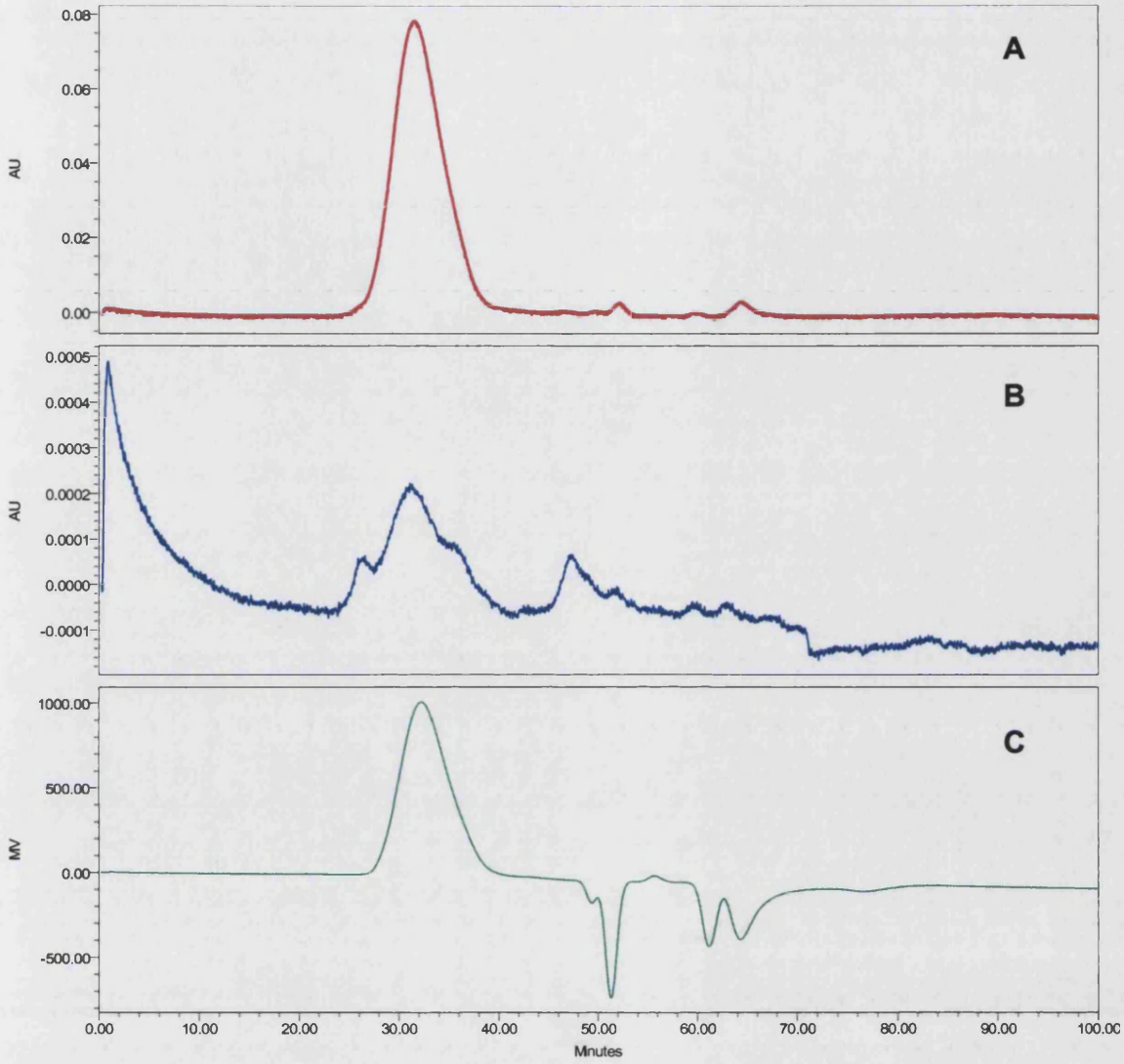

Figure 6.13 Gel Filtration profiles of *Streptococcus pneumoniae* capsular polysaccharide serotype 9V

Pneumococcal CPS serotype 9V (100μg) was eluted on a TSK G5000 column in PBS at 40°C as described in methods section 2.2.1d. The refractive index (C) of eluant was monitored together with absorbance at 205nm (A) and 280nm (B).

content by the method described in Section 2.2.3c. The assay showed there to be less than 200ng/ml of protein in 100µg/ml of CPS. Mice were only immunised with 5ug of CPS which contained approximately 10ng of protein.

In order to show that the IgG response is specifically to the CPSs and not to contaminants, a number of methods were adopted. The IgG response to 19A was shown to cross react with 19F but not to any other CPSs by ELISA. CPSs 19A and 19F are structurally similar (Lee and Fraser, 1980) so it was expected that antibodies that bind to 19A would also bind to 19F. The structures of the pneumococcal CPSs are illustrated in Figure 1.4 in the Introduction. IgG to serotypes 4 and 14, did not cross react with any of the other CPSs and were therefore serotype specific. Further, periodate oxidation of CPS 14 resulted in loss of IgG binding indicating recognition of a polysaccharide component. Oxidation of CPS 14 is known to destroy B cell epitopes. Proteins, in contrast, are periodate resistant so they would not be altered by this process. All the evidence suggests that the IgG response is pneumococcal serotype specific and not to a protein contaminant, and where there was an IgG response it was complement dependent.

As shown in section 6.3.4, the IgM response to DNP-ficoll is not complement dependent. Figure 6.9B shows the IgG response to DNP-ficoll in both Cr2-/- and C57BL/6 mice. The response in Cr2-/- is significantly reduced compared to C57BL/6 mice. The IgG response is therefore complement dependent.

6.4 Discussion

Much of our knowledge about the humoral immunity to TI-2 antigens comes from the studies of non physiological antigens. Only one recent study by Pozdnyakova and colleagues (Pozdnyakova et al., 2003), investigated the role of complement in the humoral response to a clinically relevant polysaccharide. *S. pneumoniae* is a major cause of morbidity and mortality in the paediatric population worldwide. The polysaccharide capsule protects the bacteria from innate immune defences. The host antibody response to the capsule is therefore crucial. Purified *S. pneumoniae* CPS can induce direct activation of C3 via the alterative pathway (Griffioen et al., 1991). C3d signals through CD21, which is highly expressed on MZ B cells. MZ B cells have been shown to have an important role in the response to T-independent type II antigens, by the abrogated antibody response to polysaccharides in mice that lack MZ B cells (Pyk -/- mice) (Guinamard et al., 2000). CD21 is expressed on B cells as a complement complex (CD21/CD19/CD81). Co-ligation of the B cell receptor with the complement complex by C3d attached to antigen lowers the threshold for B cell activation (Carroll, 1998). FDCs also express CD21 along with CD35. These proteins are expressed from the same locus in mice. These receptors are important for retention of complement coated antigen within the spleen (Fang et al., 1998, Qin et al., 1998). Evidence in the literature shows complement to have an important role in the host response to pneumococcal CPS. To further investigate the role of complement, the humoral response in mice deficient in complement receptors CD21 and CD35 (Cr2-/-) from M Carroll (Ahearn et al., 1996) was studied.

Prior to investigating the differences in the immune response to *S. pneumoniae* CPSs in C57BL/6 (wild type) and Cr2-/-, the nature of the IgM response in wild type mice was established. Firstly the levels of natural serum IgM antibodies against CPS in naïve wild type mice were determined. Natural serum antibodies, mostly of the IgM isotype, are produced by B1 cells. These natural antibodies appear in the absence of apparent antigenic stimulation. Natural IgM antibodies were detected to all CPSs, but levels did differ among serotypes. The presence of natural IgM to some CPSs has very low (e.g. serotypes 1 and 4). The level of natural IgM to the common teichoic acid of *S. pneumoniae* (CW-PS) was also assessed. The presence of natural IgM which bind the phosphocholine moiety of CW-PS has been shown to be protective in pneumococcal infection (Briles et al., 1981), although we detected relatively low levels of natural IgM to CW-PS compared to some CPSs. The importance of natural IgM in innate immunity to *S. pneumoniae* has been demonstrated in mice that lack secretory IgM. The presence of natural IgM to *S. pneumoniae* activates complement via the classical pathway which is one major route of clearance of *S. pneumoniae* (Brown et al., 2002).

The presence of natural IgM was also detected in Cr2-/- mice, although levels of IgM to most of the CPSs were lower in Cr2 -/- mice compared to wild type. It has been previously shown (Ahearn et al., 1996) that the Cr2-/- mice used in this work have no difference in either their total cell numbers or the ratios of peripheral mononuclear cells to granulocytes compared to wild type mice. There are no significant differences in the number of splenic

lymphocytes, or ratios of CD4 to CD8 T cells. In contrast there is a significant reduction in the B1-a (IgM^+ $CD5^+$) population of peritoneal B1 cells. Other studies have shown that there is a loss of certain B1 cell specificities and not a proportional decline in the repertoire. It was shown that the Cr2-/- mice had a similar frequency of phosphorylcholine specific CD5+ B1 cells, but a reduction in the number of intestinal ischemia-reperfusion injury specific antibodies compared to wild type mice (Fleming et al., 2002, Reid et al., 2002). CD21 and CD35 are therefore important in the maintenance of the B1 cell repertoire to some, but not all, specificities. This may explain why there is a reduction of natural antibodies to certain pneumococcal serotypes in Cr2-/- mice. Pneumococcal polysaccharides that are structurally similar like 9V and 9N, 19A and 19F all have similar levels of natural antibodies. Natural antibodies to structurally similar CPS are probably produced from the same B1 cell specificities.

After determining the levels of natural IgM, the kinetics of the primary antibody response to co-immunised CPSs in wild type mice was determined. Mice responded in a dose dependent manner to pneumococcal serotypes 6B, 14, 19A and 19F and gave an IgM response at both 7 and 21 days post immunisation. A booster immunisation given 7 days after the initial immunisation had no effect on the antibody levels to the polysaccharides, although this is not surprising considering polysaccharide antigens do not induce immunological memory. This observation is consistent with other studies which show the administration of a booster has no effect on the magnitude of the anti-pneumococcal CPS response (Aaberge et al., 1993;

Baker et al., 1971). Interestingly, the IgM response could still be detected 6 months post immunisation for serotypes 6B, 14, 19A, and 19F. This has been shown previously in BALB/c mice for pneumococcal CPSs (Aaberge et al., 1993). CPSs are high molecular weight molecules that have a long half life. This, together with their poor internalisation by B cells, results in potent persistent signalling (Mond et al., 1995a). This could explain the longevity of the antibody response.

The antibody response to pneumococcal CPSs was compared in Cr2-/- and wild type mice. For these studies, immunisations with a single rather than a mixture of CPSs were used following the discovery that the IgM antibodies generated against one CPS serotype may cross react with structurally unrelated CPSs (Figure 6.7). This could explain the difference in the IgM response to serotype 14 (Figure 6.8) when immunised alone or when co-immunised with CPSs 6B, 19A, 19F (Figure 6.3 and 6.11). When immunised alone there is no response to serotype 14 in C57BL/6 but when co-immunised there is an obvious increase in IgM levels compared to PBS-immunised mice. It is possible that when mice are co-immunised with 6B, 14, 19A and 19F that the IgM produced to 6B, 19A, 19F cross reacts with serotype 14.

The lack of response to pneumococcal CPS 14 could also be explained by a number of studies in the literature looking at conformational epitopes of pneumococcal serotype 14 (Wessels and Kasper, 1989, Laferriere et al., 1998, Zou et al., 1999). It was shown that antibodies directed against

pneumococcal serotype 14 recognise a conformational epitope fully expressed only on high molecular forms of the antigen (Wessels and Kasper, 1989). This has also been shown for group B *Streptococcus* type III (Zou et al., 1999) and group B *N. meningitidis* (Jennings et al., 1985) CPS. Pneumococcal CPS 14 is structurally related to that of group B Streptococcus type III, differing only in that the pneumococcal CPS 14 polysaccharide lacks sialic acid residues. The conformational epitope for group B *Streptococcus* type III has been shown to exist mainly in a random coil form, which structurally mimics self antigens, but it can infrequently and spontaneously form extended helices. The immune system may preferentially select these helical epitopes because they are unique to the polysaccharide (Zou et al., 1999). It is possible that this can also be applied to pneumococcal CPS 14 and the absence of an antibody response is due to the conformation of the polysaccharide. If an extended helical structure is not formed then antibodies will not recognise the conformational epitope.

The CPSs when immunised individually varied in their immunogenicity in wild type mice. Some CPSs induced a good IgM (e.g 19F, 3, and 4) response whereas others only induced low or no IgM antibodies (e.g. 9N, 9V and 14) to the CPSs. Interestingly, there was no correlation between the levels of natural IgM to CPSs and the subsequent increase in serum IgM post immunisation. Natural IgM to CPS type 4 was low but a good IgM response was observed after immunisation, whereas type 19A which also gave a good IgM response after immunisation had much higher levels of natural IgM than type 4.

In this study, only the IgM response to capsular serotype 4 was CD21/CD35 dependent. Other studies showed that complement depletion with CVF in BALB/c mice resulted in abrogation of the primary antibody response and diminished plaque forming cell (PFC) response to pneumococcal serotypes 14 (Markham et al., 1982) and 3 (Pepys, 1974). Our studies show deficiency of CD21 and CD35 to have little effect on the IgM antibody response to CPSs 14 and 3. This was consistent with studies in C3-/- mice (unpublished data from Dr. S. Zamze, Edward Jenner Institute for Vaccine Research, UK.). Possible reasons for the difference in the results could be the different mouse strains, route of immunisation or the effect of CVF on components of the immune system other than complement.

It is not known why the IgM anti-serotype 4 CPS response is dependent on complement. The only structural feature of serotype 4 that is different to the other pneumococcal CPSs is the presence a cyclic pyruvate modification, which is the immunodominant epitope (see structure Table 1.4 in the Introduction). Differential complement dependency in the humoral response to CPS may also be explained by the involvement of different B cell subsets. When human B cells were co-stimulated *in vitro* with capsular serotype 4 and anti-CD21 antibodies, the anti-CPS response was enhanced (Griffioen et al., 1992). It could be hypothesised that the IgM response to capsular serotype 4 is produced from a different subset of B cells than other pneumococcal serotypes. MZ B cells could be the major producer of antibodies to capsular serotype 4. These cells have high levels of CD21 expression. The antibody response to other pneumococcal serotypes could be from peritoneal B1 cells

which do not express CD21. Therefore in Cr2-/- mice, the antibody response to only capsular serotype 4 would be abrogated.

Pneumococcal CPS 4 has been shown in other studies to act differently to most other pneumococcal CPSs. It was found not to be recognised by the mannose receptor (Zamze et al., 2002) and other mammalian lectins such as SIGN-R1 (unpublished data from Dr. E. McGreal, Oxford University, U.K.). It could therefore be speculated that serotype 4, has different properties than other pneumococcal CPSs. A preliminary study to look at the effect of dose on the CD21/CD35 dependent response to serotype 4 (Figure 6.10) showed there to be no effect, in contrast to T-dependent antigens where complement dependency can be overcome by increasing dose (Chen et al., 2000).

Interestingly, it was shown that when mice were co-immunised with pneumococcal CPSs 6B, 14, 19A and 19F the IgM response is complement receptor dependent, but when these CPSs were immunised independently the IgM response was the same in Cr2 -/- and wild type mice. Why this is so is not known. A preliminary study using a combination of 4 other serotypes showed no difference in response in Cr2-/- or wild type mice. It is possible that the combination of 6B, 14, 19A, and 19F activate complement to a greater extent than each serotype alone or that different B cell populations respond to the different antigens as mentioned previously for serotype 4. There is no obvious reason why 6B, 14, 19A and 19F should be different to other combinations of CPSs. It is important that these studies are continued and other combinations of serotypes are considered especially because of

the possible implications this may have on multivalent polysaccharide vaccines. It is possible that a combination of CPSs may induce anti-CPS antibodies by a different mechanism from that of individual CPSs.

Also included in the study was DNP-ficoll a model T-independent type II antigen. The IgM response was found to be normal in Cr2 -/- compared to wild type but the IgG was significantly reduced in the Cr2-/- mice. These results are consistent with other studies with DNP-ficoll (Haas et al., 2002). It was for this reason that the IgG response of mice to pneumococcal CPSs was included in the study. Most of the studies in the literature showed little or no IgG response in wild type mice to pneumococcal CPSs, when administered alone without adjuvants (Aaberge et al., 1993, Aaberge and Lovik, 1996, Test et al., 2001). In the present study no IgG was detected for the following CPSs 1, 2, 3, 6B, 9V, 9N, 18C, 19F, and 23F. IgG was found in some, but not all, mice immunised with CPSs 4, 19A, and 14. The IgG response was shown probably to be specific to the polysaccharide and not to a protein contaminant within the CPS preparations (Figure 6.13). No IgG response was seen in the Cr2-/- mice which suggest that when there is an IgG response it is CD21/CD35 dependent. This study should be continued with greater number of mice to look at the frequency of an IgG response in normal mice.

In conclusion, the role of CD21/CD35 in the primary serum antibody response to *S. pneumoniae* CPS was studied. The IgM response to pneumococcal CPS 4 was shown to be dependent on CD21/CD35, whereas

there was no difference in the response of Cr2-/- mice to any of the other CPSs investigated compared to wild type mice. Similar studies in C3-/- mice illustrate the consistency of the results (unpublished data from Dr. S. Zamze, Edward Jenner Institute for Vaccine Research, UK.). There was no IgG response to most of the CPSs, only CPSs 4, 14, and 19A produced an IgG response and this was shown to be specific. It was also shown that where there was IgG response it was CD21/CD35 dependent.

Chapter Seven

Final Discussion

Chapter Seven

Final Discussion

7.1 The adjuvant properties of *Klebsiella pneumoniae* capsular polysaccharides

Many efforts are underway to find new vaccines for various pathogens. Together with these studies it is important that appropriate adjuvants are studied. Live vaccines stimulate immunity *via* transient infection caused by the replicating organism. Although these vaccines can be cost effective and easy to produce, there are serious intrinsic health risks associated with the use of live pathogens as vaccines. The more favoured type of vaccines are composed of inactivated organisms or their constituents. These non-replicating vaccines are the safest, but often lack sufficient immunogenicity and require adjuvants to elicit an adequate immune response. Currently Alum is the only licensed adjuvant in humans. Consequently the development of new adjuvants is a major goal in the vaccine field. It is important that vaccine adjuvants are defined products with low toxicity and elicit an appropriate response to protective antigens from each type of infection. One of the major aims of this thesis was therefore to further investigate potential adjuvant properties of microbial polysaccharides and the mechanisms for their biological activities.

Initial reports suggested that *K. pneumoniae* CPS extracts have immunostimulatory, anti-tumour and adjuvant properties (Nakashima, 1972;

Nakashima and Kato, 1975; Yokochi et al., 1980a; Choy et al., 1996; Ho et al., 2000). They also concluded that the CPS in these extracts was responsible for these effects, although the contents of the CPS extracts in these studies were not well defined. It is more than likely that these CPS extracts contained microbial components, such as LPS and bacterial DNA, which are reported to have strong immunostimulatory properties *via* TLR engagement. Native LPS or bacterial DNA has not been considered as adjuvant candidates due to their toxicity to cells. In order to reduce toxicity chemically altered LPS or synthetic CpG oligonucleotides are required. In contrast, CPSs are commonly used as antigens in human vaccines and are generally considered to be stable and not overtly toxic. If the reported adjuvant properties of *Klebsiella* CPS were due to CPS alone and these CPS are non-toxic to cells, then they would be desirable adjuvant candidates for use in humans. It was therefore important for our studies that the CPS was very pure and well characterised.

A successful method of *K. pneumoniae* CPS purification was established. Gel filtration chromatography was performed using a TSK G5000 column in PBS containing 0.25% (w/v) sodium deoxycholate. This method gave a highly pure CPS preparation termed CPS pool 1 which contained only trace amounts of nucleic acid, protein and LPS. Prior to purification our studies showed that CPS extracts from *K. pneumoniae* serotypes K1, K3, K55, and K52 enhanced the antibody response to CGG and therefore contained active components. Adjuvanticity was still observed independent of the TLR4 siganlling pathway and therefore of LPS. This result has confirmed that there are components in the CPS extracts other than LPS that can augment the

antibody response to CGG. Following further CPS purification the CPS pool 1 (pure high MW CPS) failed to induce an antibody response to CGG in LPS-hyporesponsive mice. This was the case for a number of different serotypes. It was therefore concluded that high MW CPS did not contribute to the adjuvant properties of CPS extracts on the enhancement of the antibody response to CGG.

Possible mechanisms to account for the observed adjuvanticity were explored. These included the induction of cytokines and stimulation of B cell proliferation. K52 and K55 CPS extracts induced the proliferation of B cells but not T cells. Both LPS and bacterial DNA were ruled out as the main contributing factors of B cell proliferation. K52 and K55 CPS extracts induced the release of TNFα, IFNγ, IL-10, and IL-6. Both proliferation and cytokine production from splenocytes was induced by CPS pool 2 but none, or very little, from CPS pool 1.

Our studies confirm that CPS extracts do have immunostimulatory properties, and establish for the first time that these properties are not only due to LPS and bacterial DNA. Studies also showed that a contribution from the high MW CPS in the extracts was unlikely. It is not clear what the active component (s) are, possibly lipopeptide or glycopeptide, which are biologically active in small quantities.

CPS extracts are able to induce antibody responses to a non-immunogenic protein *in vivo,* and *in vitro* studies have highlighted the ability of these

extracts to induce proliferation and release of various cytokines from splenocytes. Of particular interest is the release of IFNγ. IFNγ is produced predominantly by T, NK and NK T cells. IFNγ exhibits a wide array of proinflammatory activities and plays a key role in amplifying both innate and adaptive immune responses. IFNγ is a major product of Th1 cells and further skews the immune response toward a Th1 phenotype (cell-mediated immunity). IFNγ achieves this by promoting Th1 effector mechanisms such as innate cell-mediated immunity (via activation of NK cell function), specific cytotoxic activity (via T cell and APC interactions) and macrophage activation (Schroder et al., 2004). IFNγ also influences naïve CD4+ cell differentiation toward a Th1 phenotype. The induction of IFNγ from splenocytes by CPS extracts is therefore of major importance in terms of its use as an adjuvant due to the various effects IFNγ has on the immune system. It has not been shown in our studies whether the CPS extracts induce Th1 or Th2 immune responses. A Th1-skewed antibody response results in the production of IgG2a and IgG3 whereas Th2 response induces both IgE and IgG1. The antibody isotypes generated against CGG in the presence of *K. pneumoniae* CPS extracts should be determined in further investigations. Similarly, it would also be important to determine the effect of CPS extracts on the induction of cytotoxic T lymphocytes.

7.2 The role of complement in the humoral response to *Streptococcus pneumoniae* CPS

S. pneumoniae is a major cause of morbidity and mortality in the paediatric population worldwide. There is strong evidence that resistance to pneumococcal infection is mediated by the presence of antibodies against

the CPS of the bacteria. A polysaccharide vaccine consisting of the CPSs from 23 different serotypes has been used. This vaccine is successful in immunocompetent adults but is not efficacious in young children due to the T-independent nature of the CPS antigen. A protein-polysaccharide conjugate vaccine is currently licensed for use in the US. This converts the response from T-independent to T-dependent and therefore induces a primary as well as a memory response in children. It is important to continue studies that dissect the immune response to polysaccharide antigens because conjugate vaccines are not readily available in developing countries where disease incidence is highest. For example, it may be possible to induce a better response to polysaccharides with the aid of adjuvants. The main aim of this part of the thesis was to further understand factors that effect the immunogencity of polysaccharides and in particular the possible role of complement receptors in the induction of a primary anti-CPS antibody response.

Our studies focussed on a panel of capsular polysaccharides from *S. pneumoniae.* This provided a set of structurally different polysaccharides that could be compared to each other and to a model TI-2 antigen. Antibody responses to CPSs generated mostly IgM and increased in a dose-dependent manner. The use of a booster immunisation failed to have any effect on anti-CPS antibody levels. This was consistent with the lack of memory induction by these types of antigens in general. Interestingly, CPSs induced long-lived antibody responses as indicated by the presence of anti-CPS IgM antibodies in the mouse serum 6 months post immunisation.

Natural IgM could also be detected in naïve wild type mice to most of the *S. pneumoniae* CPSs, although levels of natural IgM did not determine the magnitude of antibody responses following immunisation with different CPSs.

To dissect the role of CD35 and CD21 (complement receptors 1 and 2 respectively) in the humoral response to CPSs from *S. pneumoniae,* mice deficient in these receptors (Cr2-/-) were utilised. Complement and complement receptors have been shown to be important in the antibody response to protein antigens but fewer studies have focussed on T-independent antigens. The mechanism by which complement can augment antibody responses is thought to be *via* the engagement of CD21 on B cells with complement bound antigen. CD21 is found in a complex with CD81 and CD19 and co-ligation of the BCR with this complex result in lowering of the threshold for B cell activation. Our studies focussed on a panel of clinically relevant T-independent antigens. Our first observation was that the levels of IgM in naïve mice to most of the pneumococcal CPSs tested were significantly reduced in Cr2-/- mice compared to wild type mice. Cr2-/- have been shown to have a reduced number of peritoneal B1 cells and to have lost certain specificities in the B cell repertoire (Fleming et al., 2002, Ahearn et al., 1996, Reid et al., 2002). Following immunisation with individual CPSs in both Cr2-/- and wild type mice the IgM response was similar in both groups for all CPSs except for serotype 4. The presence of IgG in the mouse sera was also investigated. IgG was detected to some CPS in wild type mice, but this was very rare. Although no IgG to the CPSs was detected in Cr2-/- mice

suggesting that the IgG response, when present, is possibly complement dependent.

It is unclear as to why pneumococcal CPS type 4 was the only CPS tested to have a complement-dependent IgM response. Subsequently, similar results were obtained using C3-/- (unpublished data from Dr. S. Zamze, Edward Jenner Institute for Vaccine Research, U.K.). In parallel with the CPSs, a model antigen, DNP-Ficoll, was used for comparison. The IgM response was normal in Cr2-/- mice but the IgG response was reduced which is consistent with what has been described in the literature (Haas et al., 2002). This highlights the differences in responses to different T-independent type II antigens, and the difference between CPS and a model antigen. MZ B cells and B1 cells are thought to be important in the response against polysaccharides. MZ B cells express CD21 but B1 cells express very little or none at all. It is possible that the B cells which are responding to type 4 are MZ B cells. This could explain why the response is complement dependent, whereas, mostly B1 cells are producing antibodies in response to the other pneumococcal CPSs. Further work to determine which B cells are producing antibodies in response to the pneumococcal CPSs could possibly provide an answer to why the IgM response to type 4 is complement dependent, these experiments are currently being carried out in the group. Interestingly, other studies have highlighted differences between type 4 CPS and other pneumococcal CPSs with regard to its lack of binding to the mannose receptor (Zamze et al., 2002) and a number of other mammalian lectins (unpublished data from Dr. E. McGreal, Oxford University, U.K.). Whether

differences in the interaction of type 4 CPS with APCs *via* lectin recognition affects the immune response remains to be determined.

7.3 Final Summary

The work presented in this thesis has provided further significant insights into the immunogenicity and potential adjuvant properties of CPS. Compared to other studies of *K. pneumoniae* CPS as adjuvants, this is the only study in which well defined products have been used and possible microbial contaminants have been ruled out. Although high MW CPS did not appear to be responsible for adjuvant or immunostimulatory properties of the CPS extracts, a component which is heat and protease resistant and potent in very small amounts may be responsible for the effects. The possible role of lower MW CPS (possibly lipidated) has not been ruled out. Further studies are continuing in the lab to define the adjuvant component(s).

Although complement receptors 1 and 2 have been shown to be important in the humoral response to TD antigens this is not the case for all TI-2 antigens. This is the first study that focussed on the use of an array of structurally different and clinically relevant TI-2 antigens. Our results have shown that only one of these have an IgM complement receptor dependent response. An IgG response to the CPS was very rare in mice used in our studies but when present it was totally dependent on the presence of complement receptors 1 and 2. The use of complement to enhance the IgG response to CPS may be an important issue in the improvement of CPS-based vaccines. The conjugation of C3 fragments to CPS, or increasing the activation of complement by CPS may represent two ways of inducing anti-CPS IgG

responses. Our studies have also highlighted the differences in humoral response to CPSs and model TI-2 antigens and that overall conclusions on CPS immunogenicity cannot always be made from studies of single antigens.

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